

ORIGINAL ARTICLE

Biomass sorghum as a novel substrate in solid-state fermentation for the production of hemicellulases and cellulases by *Aspergillus niger* and *A. fumigatus*

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Abstract**Aims:** We investigated the role of carbon and nitrogen sources in the production of cellulase and hemicellulase by *Aspergillus* strains.**Methods and Results:** The strains *Aspergillus niger* SCBM1 and *Aspergillus fumigatus* SCBM6 were cultivated under solid-state fermentation (SSF), with biomass sorghum (BS) and wheat bran (WB) as lignocellulosic substrates, in different proportions, along with variable nitrogen sources. The best SSF condition for the induction of such enzymes was observed employing *A. niger* SCBM1 in BS supplemented with peptone; maximum production levels were achieved as follows: 72 h of fermentation for xylanase and exoglucanase (300.07 and 30.64 U g⁻¹ respectively), 120 h for β -glucosidase and endoglucanase (54.90 and 41.47 U g⁻¹ respectively) and 144 h for β -xylosidase (64.88 U g⁻¹). **Conclusions:** This work demonstrated the viability of the use of BS for the production of hemi- and cellulolytic enzymes; the high concentration of celluloses in BS could be associated with the significant production of cellulases, mainly exoglucanase.**Significance and Impact of the Study:** This is the first study which presents the promising use of biomass sorghum (genetically modified sorghum to increase its biomass content) as an alternative carbon source for the production of enzymes by SSF.**Introduction**

The growing world population, the depletion of natural resources and the environmental pollution have led to the search for alternative energy sources, such as biofuels from agroindustrial by-products (Demirbas 2009). In this context, cellulosic bioethanol or second-generation ethanol (2G ethanol) is synthesized by the hydrolysis of vegetal biomass from waste products, in which complex carbohydrates from the cell wall can be converted into monosaccharides, followed by alcoholic fermentation (Baeyens *et al.* 2015).

Lignocellulose mainly consists of cellulose, hemicellulose and lignin fibres. Cellulose is a homopolymer of D-glucose residues linked by β -1,4-glycosidic bonds,

surrounded by hemicellulose and lignin. Hemicellulose consists of branched and heterogeneous polymers of pentoses, hexoses and acetylated sugars, mainly xylan, while lignin is a macromolecule of phenylpropane units linked by different types of bonds (Agbor *et al.* 2011; Gupta and Tuohy 2013). Lignocellulosic biomass is a cheap and readily available material for the production of bioethanol. However, the high content of lignin makes the biomass recalcitrant to enzymatic attacks and the subsequent hydrolysis for the production of fermentable sugars (Agbor *et al.* 2011).

Biomass sorghum (BS) is a promising lignocellulosic raw material due to its vigorous and rapid growth. It reaches a height of up to 5 m, with a high productivity and the potential to produce more than 50 t ha⁻¹ of dry

matter per half-yearly average cycle (May *et al.* 2014). Additionally, its composition resembles that of other crops conventionally studied for use in bioethanol production, such as sugarcane bagasse (SCB), although BS contains relatively lower lignin and higher hemicellulose and cellulose concentrations when compared to SCB (May *et al.* 2014). Moreover, its high biomass productivity and high amounts of cellulose and hemicellulose make BS a potential material for the application in the generation of value-added products, such as 2G ethanol and enzymes (Demirbas 2003; Horst 2017).

Cellulases and hemicellulases hydrolyse cellulose and hemicellulose in lignocellulosic materials respectively. Cellulose degradation requires the synergistic action of three classes of cellulases: endoglucanases or carboxymethylcellulases (EC 3.2.1.4), exoglucanases or avicelases (EC 3.2.1.91) and β -glucosidases or cellobiases (EC 3.2.1.21). Cellulases act by penetrating the porous regions of the cellulose fibrils (amorphous regions) and promote the depolymerization of the chains (Gupta and Tuohy 2013), while carboxymethylcellulases or CMCases hydrolyse the internal glycosidic bonds in the cellulose chains and exoglucanases act preferentially at the ends of the chains. The product of these reactions is the disaccharide cellobiose, which can be hydrolysed by β -glucosidases (Dashtban *et al.* 2009). Cellobiose is a strong inhibitor of endo- and exoglucanases, and its accumulation may limit the capacity of these enzymes to completely degrade cellulose molecules in the lignocellulosic material (Dashtban *et al.* 2009; Gupta and Tuohy 2013). The presence of β -glucosidase solves this problem, since it acts by hydrolysing cellobiose to free glucose. Among the hemicellulases, xylanases (EC 3.2.1.8) act on xylan heteropolymers and cleave glycosidic bonds, releasing xylo-oligosaccharides and xylobiose. Subsequently, β -xylosidases (EC 3.2.1.37) hydrolyse the xylo-oligosaccharides and xylobiose in free xylose (Gupta and Tuohy 2013). Nonetheless, currently, the enzymatic hydrolysis of biomass represents a challenge due to the high costs of these enzymes (Baeyens *et al.* 2015).

Alternatively, cellulases and hemicellulases can be produced by micro-organisms via solid-state fermentation (SSF), wherein the substrate provides the necessary nutrients for the microbial growth and the induction of the enzymatic production. Filamentous fungi are the most adapted organisms to be cultivated on solid substrates due to the similarity of lignocellulosic biomass to their natural habitat; they have therefore been widely studied in the field of enzyme production (Yoon *et al.* 2014; Chen *et al.* 2015; Alfenor and Molina-Jouvea 2016; Jiang *et al.* 2016). Among these fungi, several strains of *Aspergillus* have been considered good producers of enzymes for cellulose and hemicellulose degradation, such as

xylanase, β -xylosidase and β -glucosidase (dos Santos *et al.* 2015; Gomes *et al.* 2016). In this context, we cultivated *Aspergillus fumigatus* (SCBM6) and *Aspergillus niger* (SCBM1) strains under SSF, using biomass sorghum (BS) and wheat bran (WB) in different proportions as carbon sources and different nitrogen sources in order to optimize the biosynthesis of such enzymes.

Materials and methods

Chemical characterization of carbon sources

The concentrations of moisture, ash, insoluble and soluble Klason lignin, holocellulose, cellulose and hemicellulose in biomass sorghum (BS) and wheat bran (WB) were evaluated in this study. Wheat bran (WB) was also analysed for its protein content.

The moisture content was assessed according to the Technical Association of the Pulp and Paper Industry (TAPPI T264 OM-88). Ash content was measured using the TAPPI T211 OM-92 method, while Klason lignin was determined according to TAPPI T13M-54, using 72% sulphuric acid at room temperature. The holocellulose content was analysed based on the reaction between lignin, chlorine dioxide (ClO_2) and hypochlorite ion (ClO^-), produced in redox reactions of chlorite (ClO^{2-}) in acid medium (Browning 1967). The α -cellulose was determined by extraction with potassium hydroxide (KOH) from dry holocellulose, while the hemicellulose content was measured as the percentage of the difference between holocellulose and α -cellulose contents in relation to the initial dry sample mass. In addition, the lignin, cellulose and hemicellulose compositions in BS were also quantified by high-performance liquid chromatography (HPLC, Shimadzu model RID-6A, with an oven Shimadzu, model CTO-30A, a UV-vis detector Shimadzu, model SPD-10AV and an Aminex HPX 87H was used), according to Baëta *et al.* (2016). The protein content of WB was quantified by the Kjeldahl method (Kjeldahl 1883).

Chemical characterization of nitrogen sources

The quantities of carbon, hydrogen and nitrogen in BS and WB were characterized by elemental analysis in a PerkinElmer 2400 Series II CHNS/Elemental Analyzer (Brazil). The percentages of nitrogen in saline solutions containing peptone, yeast extract, ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) and urea were also determined by the same method.

Fungal strains

The fungal strains *A. niger* SCBM1 and *A. fumigatus* SCBM6 were employed as inocula in the SSF. These

strains were previously isolated from sugarcane bagasse (SCB) piles and identified by ribosomal DNA gene sequencing (dos Santos *et al.* 2015). The micro-organisms were cultured in sterile Sabouraud agar growth medium at 30°C for 7 days up to their complete sporulated stage. Afterwards, five mycelial discs of *c.* 0.5 cm in diameter each were removed from the culture medium and used as inoculum.

Enzyme production by SSF

The substrates used in the SSF were biomass sorghum (BS) and wheat bran (WB). Biomass sorghum (BS), variety LE 299, was genetically improved for the increase in its biomass content (kindly donated by the Agricultural Research Company of Minas Gerais, EPAMIG). Wheat bran (WB) was obtained from a local market in Uberlandia, Minas Gerais, Brazil. Both materials were ground in an agricultural crusher to obtain particles with a size of about 5 mm. The substrates were washed and oven-dried at 50°C for 24 h prior to the use in the SSF.

Three stages of SSF were performed (Table 1). In the first one, fungal inocula were screened for cellulase and hemicellulase production using *A. niger* SCBM1 and *A. fumigatus* SCBM6 as inoculum separately or in consortium. After the inoculum screening, the nitrogen sources ammonium sulphate [(NH₄)₂SO₄], urea, peptone and yeast extract at 0.5% were investigated. In the third stage, the carbon sources BS and WB were analysed at different proportions. The SSF was carried out in 250-mL Erlenmeyer flasks containing 5 g of substrate (BS and/or WB) and 5 ml of sterile nutrient solution with 0.5% of the nitrogen source, 0.3% of KH₂PO₄, 0.05% magnesium sulphate heptahydrate (MgSO₄ 7H₂O) and 0.05% calcium chloride (CaCl₂) per litre of distilled water (Moretti *et al.* 2012).

Each fermentation flask containing the substrates was autoclaved and further supplemented with five mycelial discs (0.5 cm in diameter) as inoculum from cultures in Sabouraud agar growth medium; the solution was brought to a volume of 20 ml by adding nutrient solution. The fermentation using both fungal strains in co-cultivation was supplied with three mycelial discs (0.5 cm of diameter) of each micro-organism per flask (total of six mycelial discs). The Fermentations 1 to 3 were conducted for 14 days and the Fermentations 4 to 11 for 7 days at 30°C. Every 24 h, 50 ml of distilled water was added to each flask and the flasks were homogenized for 1 h at 6 g and room temperature. The samples were filtered and centrifuged at 14 534 g for 10 min. The extracts were filtered through filter paper and stored at -20°C for further enzymatic assays.

Enzyme activities

The activities of β -glucosidase, carboxymethylcellulase (CMCase or endoglucanase) and avicelase (exoglucanase) as cellulases as well as xylanase and β -xylosidase as hemicellulases were investigated. The activity of β -glucosidase was quantified at 40°C using a reaction mixture containing 50 μ l of crude enzyme extract, 250 μ l of 0.1 mol l⁻¹ citrate buffer, pH 4.5 and 250 μ l of 4 mM ρ -nitrophenyl β -D-glucopyranoside (PNPG, Sigma, Aldrich, Brazil) as substrate (dos Santos *et al.* 2015). The amount of the released ρ -nitrophenol (ρ NP) was determined by spectrophotometry at 410 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μ mol of ρ NP per minute of reaction, obtained from the standard curve of ρ NP (Baffi *et al.* 2011).

The activities of CMCase and avicelase were determined by the DNS (3,5-dinitrosalicylic acid) method, in which a reaction mixture contained 30 μ l of crude enzymatic extract and 270 μ l of 1% of carboxymethylcellulose

Table 1 Solid-state fermentation (SSF) conducted in this study

	Fermentation	Fungal strain	Carbon source	Nitrogen source
First round of SSF (selection of fungal strain)	1	<i>Aspergillus niger</i> SCBM1	BS/WB (1 : 1)	(NH ₄) ₂ SO ₄ 0.5%
	2	<i>Aspergillus fumigatus</i> SCBM6	BS/WB (1 : 1)	(NH ₄) ₂ SO ₄ 0.5%
	3	<i>A. niger</i> SCBM1/ <i>A. fumigatus</i> SCBM6	BS/WB (1 : 1)	(NH ₄) ₂ SO ₄ 0.5%
Second round of SSF (selection of nitrogen source)	4	Inoculum selected in the first step	BS	(NH ₄) ₂ SO ₄ 0.5%
	5	Inoculum selected in the first step	BS	Urea 0.5%
	6	Inoculum selected in the first step	BS	Peptone 0.5%
	7	Inoculum selected in the first step	BS	Yeast extract 0.5%
Third round of SSF (selection of carbon source)	8	Inoculum selected in the first step	WB	Nitrogen source selected in the second step
	9	Inoculum selected in the first step	BS/WB (1 : 1)	Nitrogen source selected in the second step
	10	Inoculum selected in the first step	BS/WB (2 : 1)	Nitrogen source selected in the second step
	11	Inoculum selected in the first step	BS/WB (1 : 2)	Nitrogen source selected in the second step

BS, biomass sorghum; WB, wheat bran.

(CMC) or avicel, respectively, prepared in 0.1 mol l⁻¹ citrate buffer, pH 4.5 (Miller 1959). The reactions were conducted at 40°C for 10 min and stopped by the addition of 300 µl of DNS. One unit of enzymatic activity was defined as the amount of enzyme required to release 1 µmol of glucose or xylose per minute of reaction, obtained from a standard glucose or xylose curve respectively. Xylanase activity was determined by the same method used to quantify CMCase and avicelase, but using xylan as substrate (Miller 1959). The activity of β-xylosidase was determined using the same method as for β-glucosidase, but using ρ-nitrophenyl β-D-xylopyranoside (PNPX, Sigma) as substrate (dos Santos *et al.* 2015). Cellulase activity on filter paper (FPase) was assayed in a reaction mixture containing 1 ml of 0.05 mol l⁻¹ citrate buffer, pH 4.8, 0.5 ml of enzymatic extract and a filter paper tape of 1.0 × 6.0 cm. The reaction occurred for 1 h and was interrupted by the addition of 3 ml of DNS. The amount of glucose released was measured spectrophotometrically at 540 nm (Ghose 1987).

Statistical analysis

The differences between the effects of the nitrogen source used during the SSF were evaluated using the Lilliefors normality test to evaluate the normality of the data, followed by Kruskal–Wallis analysis of variance (ANOVA) and Student–Newman–Keuls test for nonparametric data, using the statistical package of the software Minitab® 14. The level of statistical significance was set at 95%.

Results

Chemical characterization of the substrate

Biomass sorghum (BS) was composed of 39.8% cellulose, 24.9% hemicellulose and 21.7% lignin, indicating that this material has a lignocellulosic structure quite similar to that of conventional SCB (Table 2). This characterizes BS as a source rich in cellulose, suggesting it can potentially be used for the production of cellulase. Wheat bran (WB) was composed of 25.41% cellulose, 26.45% hemicellulose, 25.77% lignin and 18% protein. Our results indicate that both carbon sources can be considered as good substrates for the development of micro-organisms and the production of (hemi)-cellulolytic enzymes via SSF.

Chemical characterization of nitrogen sources

All saline solutions showed slightly acid pH values, ranging from 5.0 to 6.0. The percentage of nitrogen was 21.18% in (NH)₄SO₄, 46.62% in urea, 13.32% in peptone

and 10.78% in yeast extract (Table 3), indicating that the organic complex nitrogen sources (peptone and yeast extract) presented lower nitrogen contents than the inorganic ones.

Screening for the best inoculating strain for enzyme production

In the first step of the SSF experiments, *A. niger* SCBM1 presented the highest production of β-glucosidase (Fermentation 1), with a peak of enzymatic activity of around 50.06 U g⁻¹ between 96 and 192 h of cultivation (Fig. 1a). For β-xylosidase, maximum production was observed in the SSF employing *A. niger* SCBM1 and *A. fumigatus* SCBM6 in consortium (Fermentation 3), with 145.75 U g⁻¹ after 168 h (Fig. 1b). For xylanase, a production peak (137.80 U g⁻¹) was also obtained in the fermentation carried out with both strains after 312 h of microbial growth (Fig. 1c). *Aspergillus fumigatus* SCBM6 (Fermentation 2) produced the highest amounts of avicelase (exoglucanase), with a production peak (15.69 U g⁻¹) after 192 h (Fig. 1d). For CMCase (endoglucanase), the highest activity (66.88 U g⁻¹) was observed in Fermentation 3, using both strains, after 312 h of fermentation (Fig. 1e). Based on the maximum β-glucosidase production in a shorter period of time than for the other enzymes, *A. niger* SCBM1 was selected as the best inoculum strain for the subsequent SSF experiments.

Screening for the best nitrogen source for enzyme production

In the second stage, fermentations were performed with *A. niger* SCBM1, using only BS as a carbon source and variable nitrogen sources. In this case, the highest production of β-glucosidase occurred in Fermentation 6, using peptone as a nitrogen source, with a peak of

Table 2 Chemical composition of carbon sources

Properties	Composition (%)	
	BS	WB
Humidity	3.75	9.4
Ash	4.46	1.76
Protein	–	18.0
Carbon	42.63	45.11
Nitrogen	0.84	3.69
Klason lignin	21.72	25.77
Cellulose	39.8	25.41
Hemicellulose	24.94	26.95

BS, biomass sorghum; WB, wheat bran.

Table 3 Composition of carbon, hydrogen and nitrogen and pH of saline solutions containing nitrogen sources

Sample	pH	Carbon (%)	Hydrogen (%)	Nitrogen (%)
(NH) ₄ SO ₄	5.00	–	3.02	21.18
Urea	6.01	19.98	6.66	46.62
Peptone	6.05	41.75	6.99	13.32
Yeast extract	5.68	38.36	6.72	10.78

production of 63.61 U g⁻¹ in 168 h of SSF (Fig. 2a). For β -xylosidase, fermentations performed with peptone and yeast extract obtained similar results, with values of 64.88 U g⁻¹ at 144 h and 63.95 U g⁻¹ at 168 h in Fermentations 6 and 7 respectively (Fig. 2b). The highest xylanase rates were also observed in Fermentations 6 and 7, with values of 300.07 U g⁻¹ (using peptone) and 327.37 U g⁻¹ (using yeast extract) after 72 and 96 h of cultivation respectively (Fig. 2c). The production of avicelase was only observed in Fermentation 6, which

used peptone as the only nitrogen source, with a high activity being maintained at around 30 U g⁻¹ between 48 and 120 h of fermentation (Fig. 2d). For CMCase, SSF conducted with peptone and yeast extract showed similar results, with 41.47 and 43.52 U g⁻¹ at 120 and 168 h respectively (Fig. 2e).

Screening for the best carbon source for enzyme production

In the third stage, fermentations were carried out with *A. niger* SCBM1 as inoculum, using BS and WB at different ratios (1 : 1, 1 : 2, 2 : 1 w/w) or only WB as a carbon source and peptone as the previously selected nitrogen source. An important requirement in SSF is the relationship between carbon and nitrogen in the substrate, and WB is generally widely used in SSF in combination with other carbon sources not only because it is a good inducer of the cellulolytic system, but also due to its high

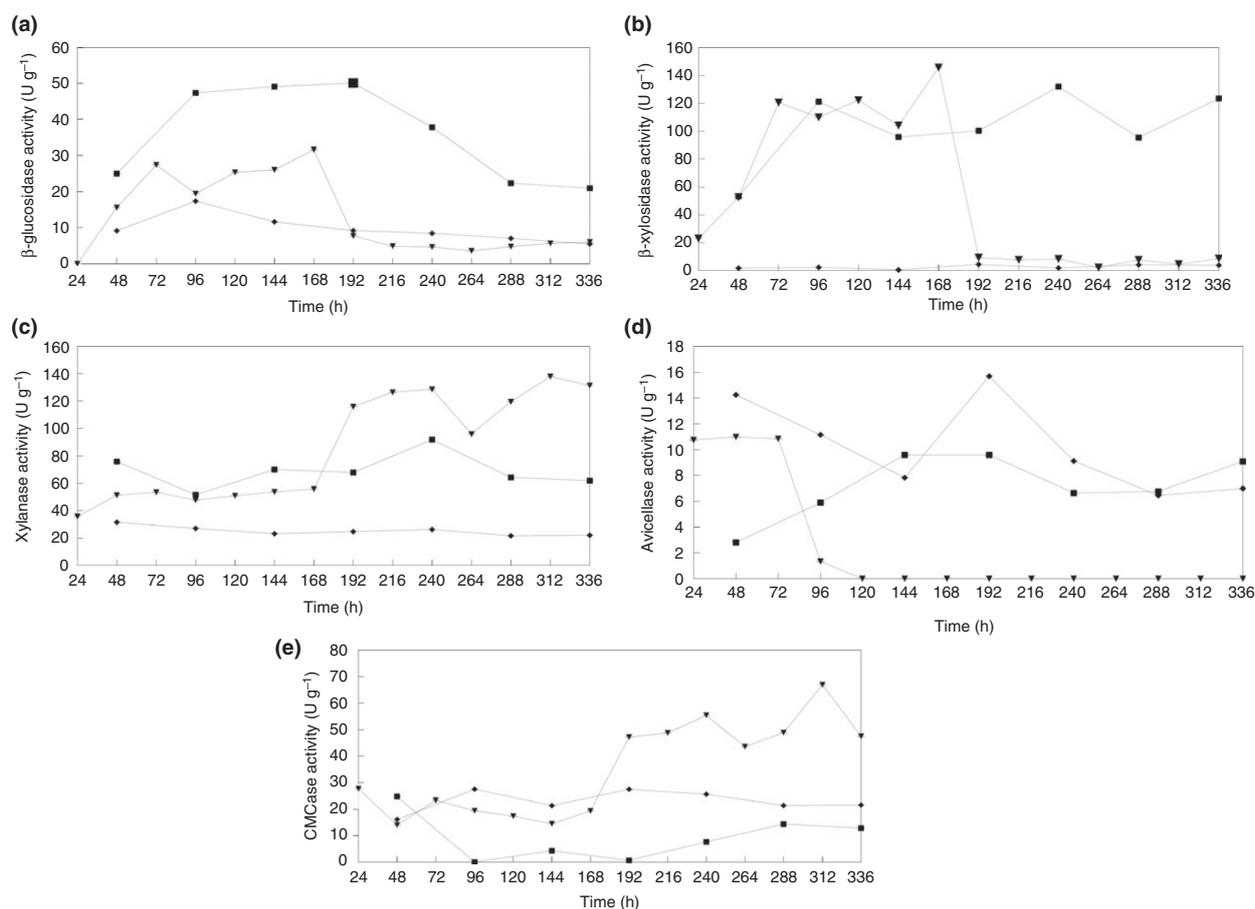


Figure 1 Production of enzymes using biomass sorghum (BS) and wheat bran (WB) (1 : 1 w/w). (■) *Aspergillus niger* SCBM1, (◆) *Aspergillus fumigatus* SCBM6 and (▼) *A. niger* SCBM1 and *A. fumigatus* SCBM6 in consortium. Enzymatic activity is expressed as U g⁻¹. Values represent mean values of experiments performed in triplicate ($P < 0.05$). (a) β -Glucosidase, (b) β -xylosidase, (c) xylanase, (d) avicelase and (e) CMCase.

protein content being, consequently, considered as an additional nitrogen source (Brijwani *et al.* 2010; Camasola and Dillon 2007). Generally, commercial WB mainly consists of nonstarch polysaccharides (41–60%), starch (10–20%) and proteins (15–20%), according to Maes *et al.* (2004).

In this step, the peak of β -glucosidase production occurred after 120 h (82.01 U g⁻¹) in Fermentation 10, when BS and WB were used at the ratio of 2 : 1 (Fig. 3a). The highest β -xylosidase biosynthesis was observed in Fermentation 9 (91.40 U g⁻¹ in 96 h), which used BS and WB at the ratio of 1 : 1 (Fig. 3b). The greater yields of xylanase were obtained after 72 and 96 h of SSF, with values of 59.23 and 59.35 U g⁻¹ in the Fermentations 8 and 9 respectively (Fig. 3c). At this stage, no carbon source induced the production of avicelase (exoglucanase). The maximum production of CMCase was obtained in Fermentation 9, employing BS and WB

as carbon sources at the proportion of 1 : 1, with 29.89 U g⁻¹ after 72 h of cultivation (Fig. 3d). We also determined FPase in the fermentation with BS and peptone (Fermentation 6); maximum FPase activity (2.11 U g⁻¹) was obtained after 144 h of fermentation (Fig. 4).

Discussion

This study was initiated by the investigation of the best inoculum for the production of cellulases and hemicellulases in biomass sorghum (BS) associated with wheat bran (WB) by SSF. For this purpose, *A. niger* SCBM1 and *A. fumigatus* SCBM6 strains were cultured separately and in co-cultivation for the production of the enzymes.

First, our results demonstrate that *A. niger* SCBM1 and *A. fumigatus* SCBM6, grown in consortium, produced high levels of most of the enzymes investigated (Fig. 1).

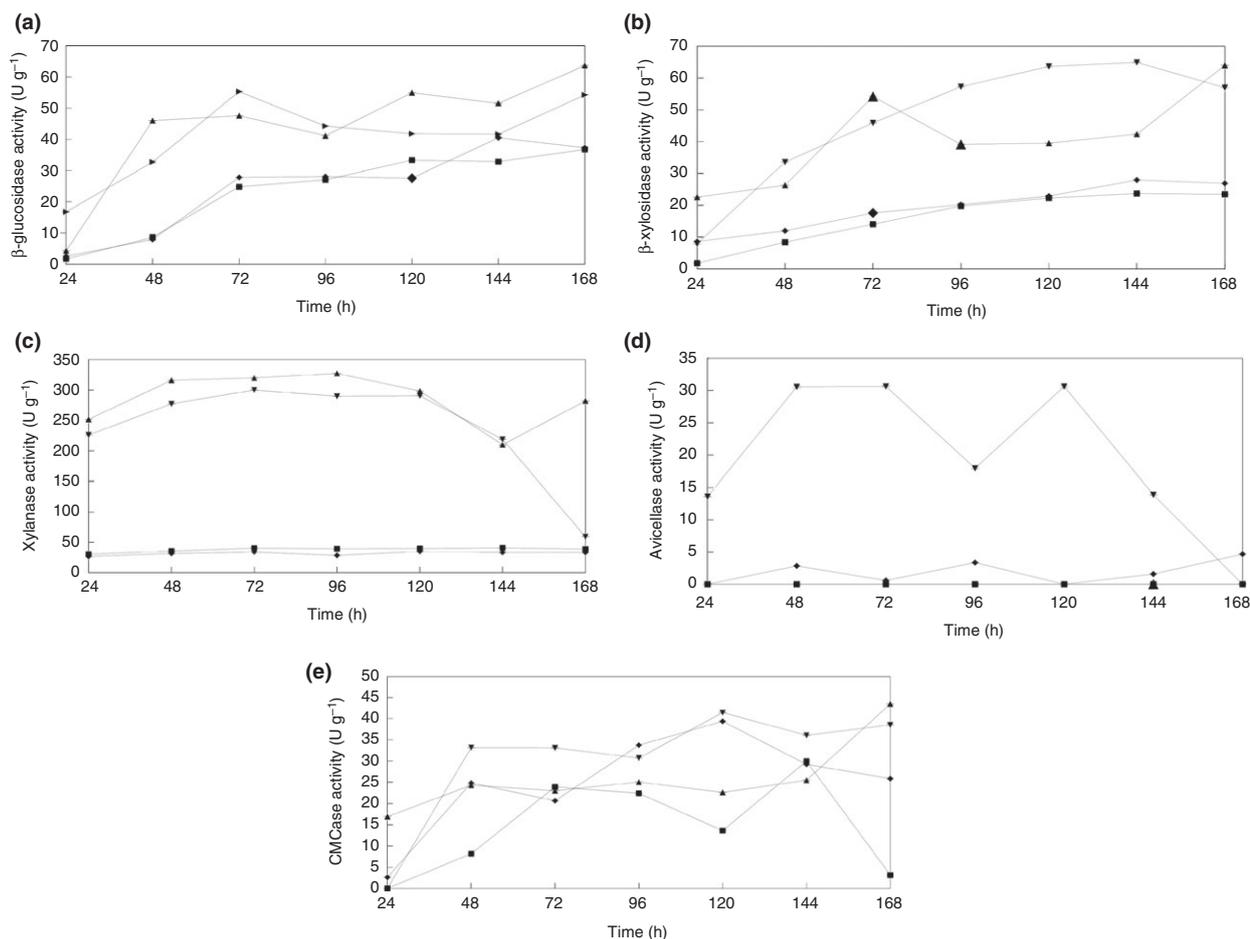


Figure 2 Production of enzymes by *Aspergillus niger* SCBM1 using biomass sorghum (BS) and variable nitrogen sources. (■) Ammonium sulphate, (◆) urea, (▲) peptone and (▼) yeast extract. Enzymatic activity is expressed as U g⁻¹. Values represent mean values of experiments performed in triplicate ($P < 0.05$). (a) β -Glucosidase, (b) β -xylosidase, (c) xylanase, (d) avicelase and (e) CMCase.

However, this was only observed with longer cultivation periods (more than 7 days of fermentation). The period until enzymatic biosynthesis is a factor of great economic importance when the large-scale commercial production is considered. An extended time for an enzymatic production can imply a higher energy expenditure to maintain the fermentation in addition to the longer period required to obtain the final product for commercialization (Klein-Marcuschamer *et al.* 2012). Thus, analysing the production of all the investigated enzymes, the fermentation with only *A. niger* SCBM1 produced high levels of the most of them, mainly β -glucosidase and β -xylosidase, but in a shorter time than the fermentation in consortium with both *Aspergillus* strains (Fig. 1a–e). Furthermore, considering only β -glucosidase, the fermentation performed with only *A. niger* SCBM1 exhibited the highest values of production (around 50.06 U g^{-1}) after between 96 and 192 h of fermentation (Fig. 1a). The enzyme β -glucosidase hydrolyses the cellobiose into free glucose molecules (Dashtban *et al.* 2009). The presence of this enzyme in the medium is of great importance to avoid the accumulation of cellobiose, which acts as a competitive inhibitor on exoglucanases, limiting their activity in cellulose depolymerization (Dashtban *et al.* 2009; Gupta and Tuohy 2013). Therefore, due to its high β -glucosidase production when compared to the SSF employing only *A. fumigatus* SCBM6 or the co-cultivation of both strains, *A. niger* SCBM1 was the strain of

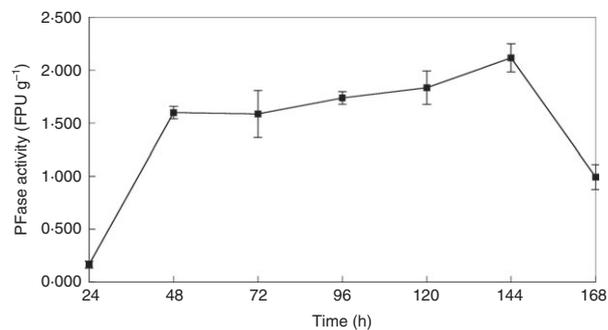


Figure 4 Production of FPase by *Aspergillus niger* SCBM1 using biomass sorghum (BS) and peptone as carbon and nitrogen sources respectively. Enzymatic activity is expressed as FPU per gram. Values represent mean values of experiments performed in triplicate ($P < 0.05$).

choice as inoculum in the following experiments, with the purpose to obtain a greater enzymatic production in a shorter period of time.

We evaluated the influence of the nitrogen source on the enzyme production by *A. niger* SCBM1 in the SSF experiments with $(\text{NH}_4)_2\text{SO}_4$, urea, peptone and yeast extract in the nutrient solution. The results of the statistical analysis indicate that there are significant differences in the β -glycosidase production between the medium containing only peptone and the medium containing $(\text{NH}_4)_2\text{SO}_4$ ($P < 0.05$) as well as the medium using urea

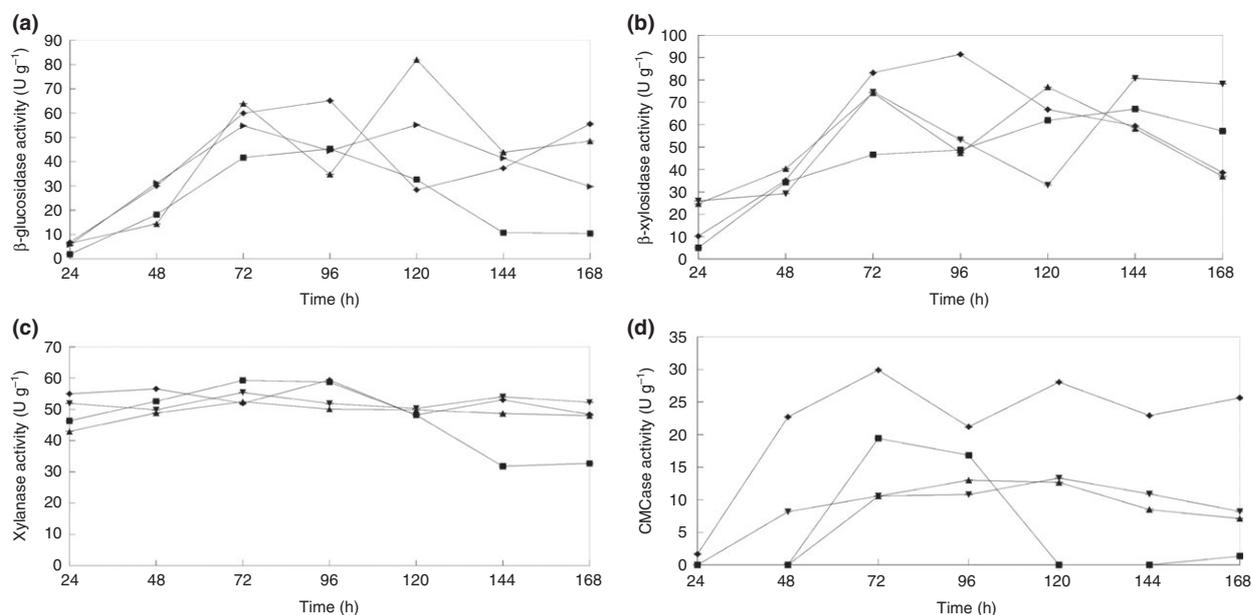


Figure 3 Production of enzymes by *Aspergillus niger* SCBM1 in variable carbon sources. (■) Wheat bran (WB), (◆) biomass sorghum and wheat bran (BS/WB 1 : 1), (▲) BS/WB (1 : 2) and (▼) BS/WB (2 : 1). Enzymatic activity is expressed as U g^{-1} . Values represent mean values of experiments performed in triplicate ($P < 0.05$). (a) β -Glucosidase, (b) β -xylosidase, (c) xylanase, (d) CMCase.

as a nitrogen source ($P < 0.05$). However, there was no significant difference ($P > 0.05$) in the enzymatic activity between the assays with peptone and yeast extract. The fermentations performed with peptone and yeast extract as the only nitrogen sources presented the highest yields for all the enzymes (Fig. 2). When the β -xylosidase was evaluated, there was no significant difference ($P > 0.05$) in the activity between the assays carried out with peptone or yeast extract after a fermentation period of 168 h. For xylanase, the highest activity was observed using yeast extract, with a significant difference ($P < 0.05$) when compared to peptone. In addition, for xylanase, yeast extract was the best nitrogen source, with a significant difference when compared to urea and $(\text{NH}_4)_2\text{SO}_4$. For CMCase activity, the selected nitrogen source was yeast extract; nevertheless, no significant difference ($P > 0.05$) was observed when compared to the production using peptone. As the assay using peptone (Fermentation 6) in the fermentation was the only one to obtain a significant production ($P < 0.05$) of avicelase (30.64 U g^{-1}), peptone was used as a nitrogen source for the following experimental stage, in which the carbon source was evaluated for the enzymatic biosynthesis (Fig. 2e). The avicelase production in the present work was higher than that (4.5 U g^{-1}) observed in a previous study via the cultivation of *Aspergillus* sp. MAM-F23 in wheat straw as substrate, without nitrogen supplementation (Abo-State *et al.* 2010).

Peptone and yeast extract are organic nitrogen sources derived from biological organic products such as milk, animal meat, soy and autolysed yeast cells (Li *et al.* 2017). Thus, in spite of their lower nitrogen content when compared to $(\text{NH}_4)_2\text{SO}_4$ and urea, other nutrients such as vitamins and mineral salts are also available in peptone and yeast extract and can positively contribute to the development of micro-organisms and the consequent induction of enzymes. This suggests that during the growth and production of enzymes, fungal strains can hydrolyse these organic nitrogen sources, releasing their mineral components and other growth factors which can then be easily incorporated into the cellular metabolism (Juwon and Emmanuel 2012). Therefore, when peptone and yeast extract were added to the culture medium instead of $(\text{NH}_4)_2\text{SO}_4$ and urea, the presence of mineral salts, vitamins and protein hydrolysates could have contributed to the increase of the microbial growth and, consequently, the enzymatic production.

The obtained results for the evaluation of the best carbon source, which included assays with BS and WB in association, indicate that the fermentation performed with BS/WB at the ratio of 1 : 1 (w/w) was most suitable to induce the overall enzymatic production in comparison to the other investigated conditions, although the

highest β -glucosidase production (82.01 U g^{-1}) was observed at the BE/WB ratio of 2 : 1 (Table 4). In general, the comparative analysis of all the fermentative conditions evaluated in the present study indicates that the SSF experiments using only BS or BS/WB (1 : 1), with peptone as a nitrogen source, presented the highest production of most of the investigated enzymes. However, for β -xylosidase, the SSF conducted with *A. niger* SCBM1 or with both *Aspergillus* strains co-cultivated in SB/WB 1 : 1 (w/w) resulted in the highest production (132.02 and 145.75 U g^{-1} respectively) when $(\text{NH}_4)_2\text{SO}_4$ was employed as a nitrogen source (Table 4). Kachlishvili *et al.* (2005) also obtained different profiles of CMCase production in different combinations of carbon and nitrogen sources. The authors observed that peptone was the most suitable nitrogen source for CMCase production when *Pleurotus dryinus* was grown on beech leaves, while $(\text{NH}_4)_2\text{SO}_4$ was the best inducer when wheat straw was employed as substrate. This leads us to infer that certain nitrogen sources may have interacting effects with certain lignocellulosic substrates and could stimulate the production of cellulases and hemicellulases via different pathways (Yoon *et al.* 2014). Thus, it is fundamental to evaluate the combination of different fungal species as well as nitrogen and carbon sources in order to improve enzyme production.

dos Santos *et al.* (2015) obtained a lower β -glucosidase production (54 U g^{-1}) using the same *Aspergillus* strains cultivated in SCB/WB (1 : 1 w/w) and $(\text{NH}_4)_2\text{SO}_4$ as carbon and nitrogen sources compared to our study which used BS or BS + WB (2 : 1 w/w) and peptone (63.61 and 82.01 U g^{-1} respectively). This suggests that BS is most suitable to induce β -glucosidase biosynthesis. In addition, BS also induced the production of endo- and exoglucanases, enzymes which were not detected by dos Santos *et al.* (2015). One explanation for this result may be the high lignin content in SCB (around 29.5% as observed by Rodrigues *et al.* 2017) in comparison to BS (21.72%), which could reduce the induction of cellulolytic enzymes. Furthermore, in the SSF carried out with $(\text{NH}_4)_2\text{SO}_4$ and only BS, the production of β -glucosidase was low (36.77 U g^{-1}), suggesting once more that peptone could potentiate the production of this enzyme (Table 4).

For β -xylosidase, dos Santos *et al.* (2015) obtained higher yields (73 U g^{-1}) than those observed in the present study (64.88 U g^{-1} with BS and peptone, 63.95 U g^{-1} with BS and yeast extract and 67.03 U g^{-1} with WB and peptone). However, when *A. niger* SCBM1 was cultivated in BS and WB (in various proportions), higher yields of this enzyme were detected than in the study by dos Santos *et al.* (2015). This result can be explained with the high content of hemicellulose in WB (26.95%), which could have contributed to the increase

Table 4 Combination of all the maximum enzymatic activities obtained in this study (expressed in U g⁻¹)

	Inoculum	Carbon source	Nitrogen source	β -glucosidase	β -xylosidase	Xylanase	CMCase	Avicelase
1	<i>Aspergillus niger</i> SCBM1	BS/WB (1 : 1)	(NH ₄) ₂ SO ₄ 0.5%	50.06	132.02	91.89	24.73	9.58
2	<i>Aspergillus fumigatus</i> SCBM6	BS/WB (1 : 1)	(NH ₄) ₂ SO ₄ 0.5%	17.35	3.71	31.41	27.46	15.69
3	<i>A. niger</i> SCBM1/ <i>A. fumigatus</i> SCBM6	BS/WB (1 : 1)	(NH ₄) ₂ SO ₄ 0.5%	31.54	145.75	137.80	66.88	10.98
4	<i>A. niger</i> SCBM1	BS	(NH ₄) ₂ SO ₄ 0.5%	36.77	23.66	41.02	30	-
5	<i>A. niger</i> SCBM1	BS	Urea 0.5%	40.51	27.91	34.97	39.41	4.67
6	<i>A. niger</i> SCBM1	BS	Peptone 0.5%	63.61	64.88	300.07	41.47	30.64
7	<i>A. niger</i> SCBM1	BS	Yeast extract 0.5%	55.26	63.95	327.37	43.52	-
8	<i>A. niger</i> SCBM1	WB	Peptone 0.5%	45.23	67.03	59.23	19.42	-
9	<i>A. niger</i> SCBM1	BS + WB 1 : 1	Peptone 0.5%	65.09	91.40	59.35	29.89	-
10	<i>A. niger</i> SCBM1	BS + WB 2 : 1	Peptone 0.5%	82.01	80.71	55.38	13.35	-
11	<i>A. niger</i> SCBM1	BS + WB 1 : 2	Peptone 0.5%	55.13	76.83	52.39	13.01	-

SSF, solid-state fermentation; BS, biomass sorghum; WB, wheat bran. Higher values of enzymatic activities are in given in bold. Outstanding line is the best fermentation condition.

of the β -xylosidase production (Table 4). Moreover, the production of this enzyme was still higher in the SSF carried out with (NH₄)₂SO₄ and BS/WB (1 : 1) as nitrogen and carbon sources (132.02 U g⁻¹) than in the SSF employing peptone and BS/WB (91.4 U g⁻¹), suggesting that the former condition is most suitable for β -xylosidase production.

In the present work, the xylanase production using BS/peptone (300.07 U g⁻¹) or BS/yeast extract (327.37 U g⁻¹) was also higher than that observed (39 U g⁻¹) by dos Santos *et al.* (2015), employing SCB/WB (1 : 1 w/w) and (NH₄)₂SO₄. In addition, BS in association with peptone induced a satisfactory production of endo- and exoglucanase, cellulases which are generally difficult to be produced at high quantities (dos Santos *et al.* 2015; Pereira *et al.* 2015). Analysing the results of the characterization of the substrates, BS presented a higher content of cellulose (39.8%) than of hemicellulose (24.94%) and lignin (21.72%), which could have probably influenced the induction of cellulase (Table 2).

Overall, we suggest the use of biomass sorghum (BS) as a promising substrate for the production of enzymes, since it was able to induce the production of both hemicellulase and cellulolytic enzymes at comparable levels to those found in previous studies carried out with sugarcane bagasse (SCB). In the present study, the use of BS and peptone as carbon and nitrogen sources represented the most ideal condition for the production of all analysed enzymes, mainly β -glucosidase, endo- and exoglucanase.

We therefore conclude that the best condition for the production of hemicellulase and cellulolytic crude enzymatic extract is represented by an SSF carried out with biomass sorghum (BS) and peptone as carbon and

nitrogen sources respectively. We suggest BS as a novel and promising raw material for SSF experiments, resulting in a high production of enzymes fundamental in the saccharification of biomass.

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Conflict of Interest

The author declares no conflict of interest.

References

- Abo-State, M.A.M., Hammad, A.I., Swelim, M. and Gannam, R.B. (2010) Enhanced Production of Cellulase(S) By *Aspergillus spp.* Isolated From Agriculture Wastes by Solid State Fermentation. *Am Eurasian J Agric. Environ Sci* **8**(4), 402–410.
- Agbor, V.B., Cicek, N., Sparling, R., Berlin, A. and Levin, D.B. (2011) Biomass pretreatment: fundamentals toward application. *Biotechnol Adv* **29**, 675–685.
- Alfenor, S. and Molina-Jouvea, C. (2016) Current status and future prospects of conversion of lignocellulosic resources to biofuels using yeasts and bacteria. *Bioprocess Biochem* **51**(11), 1747–1756.
- Ang, S.K., Shaza, E.M., Adibah, Y., Suraini, A.A. and Madihah, M.S. (2013) Production of cellulases and xylanase by

- Aspergillus fumigatus* SK1 using untreated oil palm trunk through solid state fermentation. *Process Biochem* **48**, 1293–1302.
- Baêta, B.E., Lima, D.R., Adarme, O.F., Gurgel, L.V. and Aquino, S.F. (2016) Optimization of sugarcane bagasse autohydrolysis for methane production from hemicellulose hydrolyzates in a biorefinery concept. *Bioresour Technol* **200**, 137–46.
- Baeyens, J., Kang, Q., Appels, L., Dewil, R., LV, Y. and Tan, T. (2015) Challenges and opportunities in improving the production of bio-ethanol. *Prog Energy Combust Sci* **47**, 60–88.
- Baffi, M.A., Tobal, T., Lago, J.H.G., Leite, R.S.R., Boscolo, M., Gomes, E. and Da-Silva, R.A. (2011) Novel β -glucosidase from *Sporidiobolus pararoseus*: characterization and application in winemaking. *J Food Sci* **76**, C997–C1002.
- Bansal, N., Tewari, R., Gupta, J.K., Soni, R. and Soni, S.K. (2011) A novel strain of *Aspergillus niger* producing a cocktail of hydrolytic depolymerising enzymes for the production of second generation biofuels. *Bioresour Technol* **6**(1), 552–569.
- Bansal, N., Tewari, R., Soni, R. and Soni, S.K. (2012) Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. *Waste Manage* **32**, 1341–1346.
- Brijwani, K., Oberoi, H.S. and Vadlani, P.V. (2010) Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. *Process Biochem* **45**, 120–128.
- Browning, B.L. (1967) *Methods of Wood Chemistry*. New York: Interscience Publishers, pp. 561–587.
- Camassola, M. and Dillon, A.J.P. (2007) Production of cellulases and hemicellulases by *Penicillium echinulatum* grown on pretreated sugar cane bagasse and wheat bran in solid state fermentation. *J Appl Microbiol* **103**, 2196–2204.
- Canilha, L., Chandell, A.K., Milessi, T.S.S., Antunes, F.A.F., Freitas, W.L.C., Felipe, M.G.A. and Silva, S.S. (2012) Bioconversion of Sugarcane Biomass into Ethanol: an Overview about Composition, Pretreatment Methods, Detoxification of Hydrolysates, Enzymatic Saccharification, and Ethanol Fermentation. *J Biomed Biotechnol* **2012**, 1–15.
- Chen, H., Zhou, D., Zhang, S. and Chen, J. (2015) Macroalgae for biofuels production: progress and perspectives. *Renew Sust Energy Rev* **47**, 427–437.
- Chinedu, N.S., Nwinyi, O.C. and Okochi, V.I. (2008) Growth and cellulase activity of wild-type *Aspergillus niger* ANL301 in different carbon sources. *Can J Pure & Appl Sci* **2**, 357–362.
- Dashtban, M., Schraft, H. and Qin, W. (2009) Fungal Bioconversion of Lignocellulosic Residues; Opportunities & Perspectives. *Int J Biol Sci* **5**, 578–595.
- Demirbas, A. (2003) Relationships between lignin contents and fixed carbon contents of biomass samples. *Energy Convers Manage* **44**, 1481–1486.
- Demirbas, A. (2009) Biofuels securing the planet's future energy needs. *Energy Convers Manage* **50**, 2239–2249.
- Ghose, T.K. (1987) Measurement of cellulase activities. *Pure Appl Chem* **59**, 257–268.
- Gomes, A.F.S., Santos, B.S.L., Franciscan, E.G. and Baffi, M.A. (2016) Substrate and temperature effect on xylanase production by *Aspergillus fumigatus* using low cost agricultural wastes. *Bioscience J* **32**, 915–921.
- Guimarães, C.C., Simeone, M.L.F., Parrela, R.A.C. and Sena, M.M. (2014) Use of NIRS to predict composition and bioethanol yield from cell wall structural components of sweet biomass sorghum. *Microchem J* **117**, 194–201.
- Gupta, V.K. and Tuohy, M.G. (2013) *Biofuel Technologies – Recent Developments 1*. Berlin: Springer, p. 533.
- Horst, D.J. (2017) Lignin as alternative fuel: an estimate of the thermal energy generation potential from Brazilian crops. *Environ Prog Sustain Energy* **36**, 717–722.
- Jiang, Y., Duarte, A.V., Brink, J., Wiebenga, A., Wang, C., Vries, R.P., Zhou, Z. and Benoit, I. (2016) Enhancing saccharification of wheat straw by mixing enzymes from genetically-modified *Trichoderma reesei* and *Aspergillus niger*. *Biotech Lett* **38**, 65–70.
- Jun, H., Guangye, H. and Daiwen, C. (2013) Insights into enzyme secretion by filamentous fungi: comparative proteome analysis of *Trichoderma reesei* grown on different carbon sources. *J Proteomics* **89**, 191–201.
- Juwon, A.D. and Emmanuel, O.F. (2012) Experimental Investigations on the Effects of Carbon and Nitrogen Sources on Concomitant Amylase and Polygalacturonase Production by *Trichoderma viride* BITRS-1001 in Submerged Fermentation. *Biotechnol Res Int* **2012**, 1–8.
- Kachlishvili, E., Penninckx, M.J., Tsiklauri, N. and Elisashhvili, V. (2005) Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. *World J Micro & Biotech* **22**, 391–397.
- Kjeldahl, J. (1883) Neue Methode zur Bestimmung des Stickstoffs in organischen Körpern. *Liste Chemischer Fachzeitschriften* **22**, 366–383.
- Klein-Marcuschamer, D., Oleskowicz-Popiel, P., Simmons, B.A. and Blanch, H.W. (2012) The Challenge of Enzyme Cost in the Production of Lignocellulosic Biofuels. *Biotechnol Bioeng* **104**(4), 1083–1087.
- Li, Z., Wang, D. and Shi, Y.C. (2017) Effects of nitrogen source on ethanol production in very high gravity fermentation of corn starch. *J Taiwan Inst Chem Eng* **70**, 229–235.
- Maes, C., Vangeneugden, B. and Delcour, J.A. (2004) Relative activity of two endoxylanases towards water-unextractable arabinoxylans in wheat bran. *J Cereal Sci* **39**, 181–186.
- May, A., Parrela, R.A.C., Damasceno, C.M.B. and Simeone, M.L.F. (2014) Sorgo como matéria-prima para produção de bioenergia: etanol e cogeração. *Informe Agropecuário* **35** (278), 73–81.
- Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**, 426–428.

- Moretti, M.M.S., Bocchini-Martins, D.A., Da-Silva, R., Rodrigues, A., Sette, L. and Gomes, E. (2012) Selection of thermophilic and thermotolerant fungi for the production of cellulases and xylanases under solid-state fermentation. *Braz J Microbiol* **106**, 2–1071.
- Pandey, A.K., Edgard, G. and Negi, S. (2016) Optimization of concomitant production of cellulase and xylanase from *Rhizopus oryzae* SN5 through EVOP-factorial design technique and application in Sorghum Stover based bioethanol production. *Renew Energy* **98**, 51–56.
- Pereira, J.C., Marques, N.P., Rodrigues, A., Brito de Oliveira, T., Boscolo, M., Silva, R., Gomes, E. and Martins, D.A.B. (2015) Thermophilic fungi as new sources for production of cellulases and xylanases with potential use in sugarcane bagasse saccharification. *J Appl Microbiol* **118**, 928–939.
- Rodrigues, P.O., dos Santos, B.V., Costa, L., Henrique, M.A., Pasquini, D. and Baffi, M.A. (2017) Xylanase and β -glucosidase production by *Aspergillus fumigatus* using commercial and lignocellulosic substrates submitted to chemical pre-treatments. *Ind Crops Prod* **95**, 453–459.
- dos Santos, B.S.L., Gomes, A.F.S., Franciscon, E.G., Oliveira, J.M. and Baffi, M.A. (2015) Thermotolerant and mesophylic fungi from sugarcane bagasse and their prospection for biomass-degrading enzyme production. *Braz J Microbiol* **46**, 903–910.
- Sharma, R., Rawat, R., Bhogal, R.S. and Oberoi, H.S. (2015) Multi-component thermostable cellulolytic enzyme production by *Aspergillus niger* HN-1 using pea pod waste: appraisal of hydrolytic potential with lignocellulosic biomass. *Process Biochem* **50**, 696–704.
- Tiwari, R., Nain, P.K.S., Singh, S., Adak, A., Saritha, M., Rana, S., Sharma, A. and Nain, L. (2015) Cold active holocellulase cocktail from *Aspergillus niger* SH3: process optimization for production and biomass hydrolysis. *J Taiwan Inst Chem Eng* **56**, 57–66.
- Yoon, L.W., Ang, T.N., Ngho, G.C. and Chua, A.S.M. (2014) Fungal solid-state fermentation and various methods of enhancement in cellulase production. *Biomass Bioenerg* **67**, 319–338.