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Potential of Mangrove-Associated Endophytic Fungi for Production of Carbohydrolases with High Saccharification Efficiency

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Abstract The endophytic fungi represent a potential source of microorganisms for enzyme production. However, there have been only few studies exploiting their potential for the production of enzymes of industrial interest, such as the (hemi)cellulolytic enzymatic cocktail required in the hydrolysis of lignocellulosic biomass. Here, a collection of endophytic fungi isolated from mangrove tropical forests was evaluated for the production of carbohydrolases and performance on the hydrolysis of cellulose. For that, 41 endophytic strains were initially screened using a plate assay containing crystalline cellulose as the sole carbon source and the selected strains were cultivated under solid-state fermentation for endoglucanase, β-glucosidase, and xylanase enzyme quantification. The hydrolysis of a cellulosic material with the enzymes from endophytic strains of the *Aspergillus* genus resulted in glucose and conversion values more than twofold higher than the reference strains (*Aspergillus niger* F12 and *Trichoderma reesei* Rut-C30). Particularly, the enzymes from strains *A. niger* 56 (3) and *A. awamori* 82 (4) showed a distinguished saccharification performance, reaching cellulose conversion values of about 35% after 24 h. Linking hydrolysis performance to the screening steps played an important role towards finding potential fungal strains for producing enzymatic cocktails with high saccharification efficiency. These results indicate the potential of mangrove-associated endophytic fungi for production of carbohydrolases with efficient performance in the hydrolysis of biomass, thus contributing to the implementation of future biorefineries.

Keywords Endophytic fungi · Enzymes · Cellulose · Enzymatic hydrolysis · Biorefinery

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Introduction

The bioconversion of lignocellulosic biomass into fuels, chemicals, and novel materials has been considered a potential strategy for the implementation of future biorefineries [1–4]. Among the greatest challenges faced in the application of such bioprocess are the high cost of the enzymes and the high enzyme loadings required for an efficient conversion of the lignocellulosic materials into simple sugars [5–7]. In order to overcome these techno-economic limitations, the production of enzymes on-site may be a potential approach to reduce the overall costs, including those related to transportation and storage, and therefore has been investigated employing a wide range of raw materials and fungal strains [8–14].

Multiple enzymes are required for the complete hydrolysis of lignocellulosic materials, including cellulases, hemicellulases, pectinases, ligninases, and other accessory enzymes [15, 16]. This enzymatic complex is produced by a wide variety of microorganisms (bacteria and fungi). However, the aerobic fungi are especially recognized for their high growth and protein secretion rates [17–20]. Several filamentous fungi have been used for the industrial production of cellulolytic cocktails. Among them, *Trichoderma* and *Aspergillus* strains are considered the workhorses, presenting good fermentation characteristics, such as high protein secretion rates and ability to produce a wide range of extracellular enzymes [14, 21, 22]. Nevertheless, enzyme-prospecting research continues to identify opportunities to enhance the activity of enzyme preparations by supplementation with enzymatic diversity from other microbes [10, 23–27].

The endophytic fungi represent a potential source of microorganisms for enzyme production. Endophytic fungi inhabit the internal tissues of plants without causing any negative effects, being considered a potential source of novel biochemical compounds for different biotechnological applications [25, 28–32]. The mangrove-associated endophytic fungi are of particular interest due to their natural adaptation to the extreme environment of the mangrove ecosystem situated between land and sea, which makes them as a biodiversity hotspot for product discovery [31, 33]. This extreme environment found in mangrove forests promotes the adaptation of plants and microbial communities to conditions of high salinity, tidal flooding, strong winds, and high temperatures [28, 31]. A comparative study of the diversity of endophytic fungal communities isolated from the leaves and branches of trees inhabiting two mangroves in the state of Sao Paulo, Brazil, revealed a large reservoir of fungal diversity [28, 31]. Moreover, endophytic fungi possess the two types of extracellular enzymatic systems necessary to degrade the vegetal biomass, the hydrolytic, and the oxidative system [30]. Therefore, it is of great interest to further explore the potential of mangrove-associated endophytic fungi for (hemi)cellulolytic enzyme production.

Here, the collection of endophytic fungi isolated from the Brazilian mangrove forests was evaluated for the potential of (hemi)cellulolytic enzyme production. For that, 41 fungal strains were initially screened using a plate assay and the selected strains were further evaluated for the production of cellulases and xylanase enzymes by cultivation under solid-state fermentation. The superior performance of the enzymatic cocktails from the endophytic fungi in the hydrolysis of a cellulosic material could be demonstrated by comparison with reference strains.

Materials and Methods

Fungal Strains

The collection of wild-type endophytic fungi strains used in this work is maintained by the Laboratory of Microbiology and Biomolecules (Center of Biological Sciences and Health, UFSCar, São Carlos, Brazil). The fungi were originally isolated from plant species found in mangrove forests in the State of São Paulo, Brazil [28]. A total of 41 endophytic fungi of the genus *Aspergillus*, *Diaporthe*, *Fusarium*, *Hypocrea*, *Penicillium*, and *Xylaria* were selected to be evaluated in terms of their potential to produce (hemi)cellulolytic enzymes. As reference strains, the hyper-cellulolytic mutant *Trichoderma reesei* Rut-C30 was purchased from the Centre for Agricultural Bioscience International (CABI) culture collection in the UK (IMI number: 345108) and the *Aspergillus niger* F12 was obtained from the Embrapa Food Technology collection (Rio de Janeiro, Brazil). All isolates were activated in potato-dextrose-agar (PDA) medium plates by incubation for 7 days at 28 °C.

Screening of Endophytic Fungal Strains in Selective Medium

A plate assay was used for an initial qualitative screening step of the 41 endophytic fungal strains. For that, the strains were inoculated in plates containing a synthetic medium and crystalline cellulose Avicel (Fluka Biochemika, Switzerland) as the only source of carbon [11]. After inoculation, the strains were incubated at 28 °C for 7 days. The strains that presented a distinguished growth on the Avicel plate-assay were selected for the following step of the study in which cultivations were carried out under solid-state fermentation.

Solid-State Fermentation

Solid-state fermentation cultivations were carried out in 250-mL Erlenmeyer flasks containing 10 g of wheat bran with a moisture level of 60% (adjusted with 0.9% (*w*:*v*) ammonium sulfate solution in 0.1 mol/L HCl), as described by [34]. The flasks were sterilized and then inoculated with a suspension of 10^7 spores per gram of solid medium for the sporulating strains or five pieces (8 mm) of potato dextrose agar for the non-sporulating strains. A initial set of cultivations was carried out at 32 °C, with samples removed with 24-h intervals for up to 120 h for the six representative strains selected in the plate-assay screening (*Aspergillus niger* 56 (3), *Hypocrea lixii* 1.14, *Penicillium* sp. 60 (4), *Diaporthe* sp. 37 (4), *Xylaria enteroleuca* 39.3 (1), and *Fusarium* sp.21.5). From this set of time-course experiments, the cultivation time for maximum enzymatic activity was selected for each specific genus. In the second set of cultivations, all the other strains from each genus were cultivated during the previously selected period of time. For all the cultivations, after the incubation period, the enzymes were extracted by adding 0.2 mol/L acetate buffer at pH 4.5. The samples were stirred at 120 rpm for 40 min and the enzymatic solution was recovered by filtration. The recovered enzyme extracts were stored at − 18 °C for further analysis. All cultivations were carried out in triplicate, and the data were calculated as means ± standard deviations.

Enzymatic Activities

Endoglucanase and β-glucosidase activities were assayed in the presence of carboxymethylcellulose (CMC) (Sigma, USA) and cellobiose (Sigma, USA), respectively, using the standard method proposed by Ghose [35]. In the case of β-glucosidase activity, quantification of the glucose released was performed with an enzymatic kit for glucose measurement (Doles, Brazil). Xylanase activity was measured using the method described by [36]. The reducing sugar concentration was determined by the DNS method [37]. All enzymatic analyses were carried out in duplicate. One unit of enzymatic activity corresponded to 1 μmol of reducing sugars released per minute of reaction.

Enzymatic Hydrolysis of Cellulose

The enzymatic hydrolyses of cellulose (Celluflok 200, Brazil) were performed in 5-mL tubes containing 4 mL of the crude enzymatic extracts and 200 mg of cellulose, for a solids loading of 5% (*w*/*v*). The reaction mixtures were incubated for 24 h at 50 °C in an incubator with rotary mixing at 30 rpm. Samples were withdrawn after 24 h for quantification of the glucose released using an enzymatic kit (Doles, Brazil) and reducing sugars by the DNS method [37]. The enzymatic conversion of cellulose was calculated using Eq. 1:

$$Conversion\ (\%) = \left[\frac{\left(m_{glucose}^{24h} - m_{glucose}^{0h}\right)}{\left(m_{cellulose}^{0h}\right) \times 1.11}\right] \times 100 \quad (1)$$

Where *m* is the mass of either glucose or cellulose and the value 1.11 is the theoretical yield factor for the enzymatic conversion of cellulose to glucose. All the experiments were carried out in triplicate, and the data were calculated as means ± standard deviations.

Results and Discussion

Screening of Endophytic Fungal Strains

Plate-assay screenings can be very useful as an initial tool for selection of cellulolytic enzyme-producing strains from large microbial collections. Table 1 presents the details of the 41 endophytic strains originally isolated from the mangroves of the state of São Paulo [28] and their respective results in terms of growth on agar plates containing crystalline cellulose (Avicel) as the only source of carbon. An expressive total of 36 strains (88%) showed growth on the Avicel medium (Fig. 1), indicating the potential of these microorganisms to efficiently degrade cellulosic materials.

The procedure used to prepare Avicel employing dilute-acid treatment removes the hemicellulose and most of the amorphous fraction of the cellulose fiber, resulting in a microcrystalline source of nearly pure cellulose [17]. This crystalline nature of Avicel requires that a synergistic action of several different cellulolytic enzymes, including endoglucanases, exoglucanases, and β-glucosidases, for the hydrolysis of the cellulose into soluble simple sugars that could be metabolized by the microorganism [15, 17, 38]. Therefore, the expressive growth of these 36 endophytic strains in the selective medium with Avicel as the sole source of

Table 1 Identification of endophytic strains and results of plate assay in the Avicel medium

	Taxonomic identification	Identification code of the isolate	Host plant species	Plant tissue	Avicel culture
1	*Aspergillus awamori*	82 (4)	*Laguncularia racemosa*	Leaf	Positive
2	*Aspergillus niger*	56 (3)	*Rhizophora mangle*	Leaf	Positive
3	*Aspergillus awamori*	108 (4)	*Avicennia nitida*	Leaf	Positive
4	*Aspergillus awamori*	09 (4)	*Avicennia nitida*	Branch	Negative
5	*Diaporthe stewartti*	10 (3)	*Laguncularia racemosa*	Branch	Positive
6	*Diaporthe phaseolorum*	97.4 (1)	*Avicennia nitida*	Leaf	Positive
7	*Diaporthe stewartti*	12.2 (1)	*Laguncularia racemosa*	Branch	Positive
8	*Diaporthe* sp.	9 (3)	*Laguncularia racemosa*	Branch	Positive
9	*Diaporthe* sp.	57 (4)	*Laguncularia racemosa*	Branch	Positive
10	*Diaporthe* sp.	94 (4)	*Avicennia nitida*	Branch	Positive
11	*Diaporthe phaseolorum*	51.5 (1)	*Rhizophora mangle*	Leaf	Positive
12	*Diaporthe* sp.	37 (4)	*Rhizophora mangle*	Branch	Positive
13	*Diaporthe* sp.	30 (3)	*Avicennia nitida*	Leaf	Positive
14	*Diaporthe* sp.	67.1 (1)	*Avicennia nitida*	Branch	Positive
15	*Diaporthe* sp.	5.1 (1)	*Laguncularia racemosa*	Branch	Positive
16	*Diaporthe phaseolorum*	98 (3)	*Avicennia nitida*	Branch	Negative
17	*Diaporthe* sp.	6 (4)	*Avicennia nitida*	Branch	Negative
18	*Fusarium camptoceras*	90 (3)	*Avicennia nitida*	Branch	Positive
19	*Fusarium* sp.	63.1	*Laguncularia racemosa*	Branch	Positive
20	*Fusarium* sp.	21.5	*Laguncularia racemosa*	Leaf	Positive
21	*Fusarium chlamydosporum*	75 (3)	*Laguncularia racemosa*	Leaf	Positive
22	*Fusarium* sp.	16.1	*Rhizophora mangle*	Branch	Positive
23	*Fusarium* sp.	21.2	*Laguncularia racemosa*	Leaf	Positive
24	*Fusarium* sp.	21.3	*Laguncularia racemosa*	Leaf	Positive
25	*Fusarium sambucinum*	3 (3)	*Laguncularia racemosa*	Branch	Positive
26	*Hypocrea lixii*	68 (4)	*Laguncularia racemosa*	Branch	Positive
27	*Hypocrea lixii*	1.16	*Rhizophora mangle*	Branch	Positive
28	*Hypocrea lixii*	12.6	*Laguncularia racemosa*	Leaf	Positive
29	*Hypocrea lixii*	1.14	*Laguncularia racemosa*	Branch	Positive
30	*Hypocrea lixii*	48 (4)	*Rhizophora mangle*	Branch	Positive
31	*Hypocrea lixii*	47 (4)	*Rhizophora mangle*	Branch	Positive
32	*Hypocrea koningii*	44 (4)	*Rhizophora mangle*	Branch	Positive
33	*Hypocrea virens*	89 (3)	*Avicennia nitida*	Leaf	Positive
34	*Hypocrea koningii*	36.3 (1)	*Avicennia nitida*	Branch	Negative
35	*Penicillium minioluteum*	24 (4)	*Rhizophora mangle*	Branch	Positive
36	*Penicillium sp.*	62 (4)	*Laguncularia racemosa*	Branch	Positive
37	*Xylaria enteroleuca*	53.2 (1)	*Avicennia nitida*	Leaf	Positive
38	*Xylaria polymorpha*	47.1 (1)	*Rhizophora mangle*	Leaf	Positive
39	*Xylaria enteroleuca*	33.3 (1)	*Avicennia nitida*	Branch	Negative

Table 1 (continued)

	Taxonomic identification	Identification code of the isolate	Host plant species	Plant tissue	Avicel culture
40	*Xylaria enteroleuca*	39.3 (1)	*Rhizophora mangle*	Leaf	Positive
41	*Xylaria enteroleuca*	106 (4)	*Avicennia nitida*	Branch	Positive

carbon suggests that these strains are able to produce the arsenal of enzymes required for its degradation into simple sugars.

In terms of the different endophytic genera evaluated here (*Aspergillus*, *Hypocrea*, *Penicillium*, *Fusarium*, *Xylaria*, and *Diaporthe*), all the eight strains of the *Fusarium* genus showed positive result for growth on Avicel plates. The strains of the genera *Hypocrea*, *Aspergillus*, and *Xylaria* showed one negative result each, while the strains of the *Diaporthe* genus showed two negative results for growth on Avicel plate. Interestingly, all the five negative results for growth on Avicel were from fungi strains isolated from the branch of the host plant. Also, an unexpected negative result was observed for the strain *A. awamori* 09 (4), since the genus *Aspergillus* is widely recognized as a good producer of biomass-degrading enzymes [39, 40]. Recently, this *A. awamori* 09 (4) strain was selected for its distinguished performance for organic acid production [32]. Therefore, this finding can indicate that this particular *Aspergillus* strain has a different preferential metabolic pathway.

In a previous screening study of 78 *Trichoderma* strains, an assessment of fungal growth on plates containing Avicel as the sole carbon source revealed that 49 strains (62.8% of all strains evaluated) were able to hydrolyze this cellulosic substrate and exhibited obvious growth [11]. In another study, 64 fungal isolates were screened for cellulolytic activities using a selection

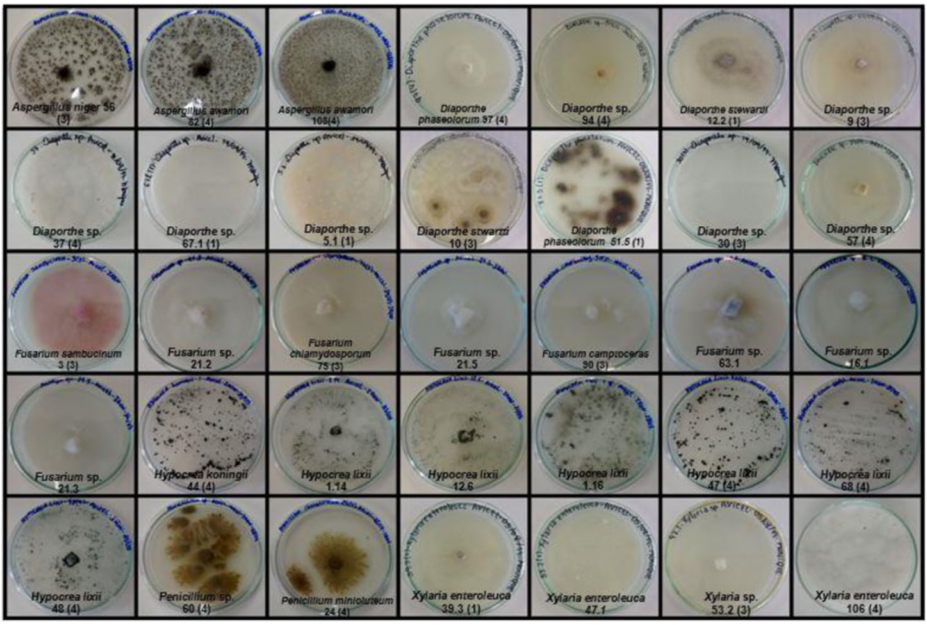

Fig. 1 Endophytic fungi strains of the genus *Aspergillus*, *Hypocrea*, *Penicillium*, *Fusarium*, *Xylaria*, and *Diaporthe* that showed a positive result in the screening plate assay for growth on crystalline cellulose as the sole carbon source

based on the growth on Avicel and CMC (carboxymethylcellulose) [41]. Of the 64 strains included in the screening, 25 were chosen as they presented good growth on these commercial substrates, with slightly better results obtained using CMC, which is a soluble source of cellulose. Regarding the screening of endophytic fungal strains for cellulolytic enzyme production, a study reported that of a total of 120 fungal strains evaluated, only 35 strains (29%) were able to both grow and produce halos in a plate assay using a selected medium [25]. Thus, the relatively high percentage of positive strains (88%) obtained in this present study indicates the potential of these endophytic strains for cellulolytic enzyme production.

Time Profile of (Hemi)Cellulolytic Enzyme Production Under SSF

Six endophytic strains from different genus were selected based on their previous results on the screening plate assay for Avicel growth for an evaluation of the solid-state fermentation (SSF) cultivation period required for maximum enzyme production. For that, the time profile of endoglucanase, xylanase, and β-glucosidase enzyme production by the selected strains of the genus *Aspergillus*, *Hypocrea*, *Penicillium*, *Fusarium*, *Xylaria*, and *Diaporthe* cultivated under SSF was evaluated for up to 120 h (Fig. 2).

In industrial processes for enzyme production, it is desirable to achieve higher production in a reduced time, in order to save costs [21]. Therefore, the time of cultivation chosen for each strain was defined as the minimum period that a considerable production was obtained for most of the enzymes quantified. In general, the evaluation of the best cultivation time for each genus was based on the maximum production of endoglucanase, followed by xylanase and β-glucosidase enzymes. Such time selection criteria had to be adopted in face of the large number of potential strains to be evaluated.

According to Fig. 2, the endophytic strain of *Aspergillus niger* 56 (3) produced a significant amount of endoglucanase (57 U/g) and xylanase (455 U/g) enzymes after 48 h of cultivation, while β-glucosidase enzyme production continued to increase up to 120 h. Following our time selection criteria, the other strains of the *Aspergillus* genus were cultivated for 48 h for their evaluation for enzymatic production under SSF. In a previous study using *A. niger* to produce cellulolytic enzymes under SSF, it was reported a maximum production of level 14 U/g of endoglucanases in 72 h [42]. In the case of the endophytic strain *A. niger* 56 (3), a fourfold value of endoglucanase activity was obtained in 48 h of cultivation, thus indicating the potential of this endophytic fungal strain.

As for the endophytic strain of the genus *Hypocrea*, the maximum production of endoglucanase was between 72 and 96 h of SSF cultivation, while for xylanases, it was between 48 and 72 h (Fig. 2). In terms of β-glucosidase, the production of this enzyme by *Hypocrea* was very low, as it has been previously reported for this genus [17, 43]. Therefore, a cultivation time of 72 h was selected for evaluation of the other endophytic *Hypocrea* strains. Florencio et al. [11] evaluated the enzymatic production of strains of the genus *Trichoderma* and found that the maximum endoglucanase production of 25.93 U/g was obtained after 8 days of SSF cultivation, which also indicates the potential of the endophytic fungal strain.

The production of both endoglucanase and xylanase enzymes by the *Penicillium* endophytic strain was relatively low and varied slightly during the cultivation period (Fig. 2). Therefore, the selection of the maximum production time for the *Penicillium* genus was based on the β-glucosidase production, which was higher (64 IU/g) in 72 h of cultivation. As for the other three genus evaluated, the cultivation time selected was 96 h for the genus *Xylaria* and 72 h for both *Diaporthe* and *Fusarium* based on the activity values presented in Fig. 2. These

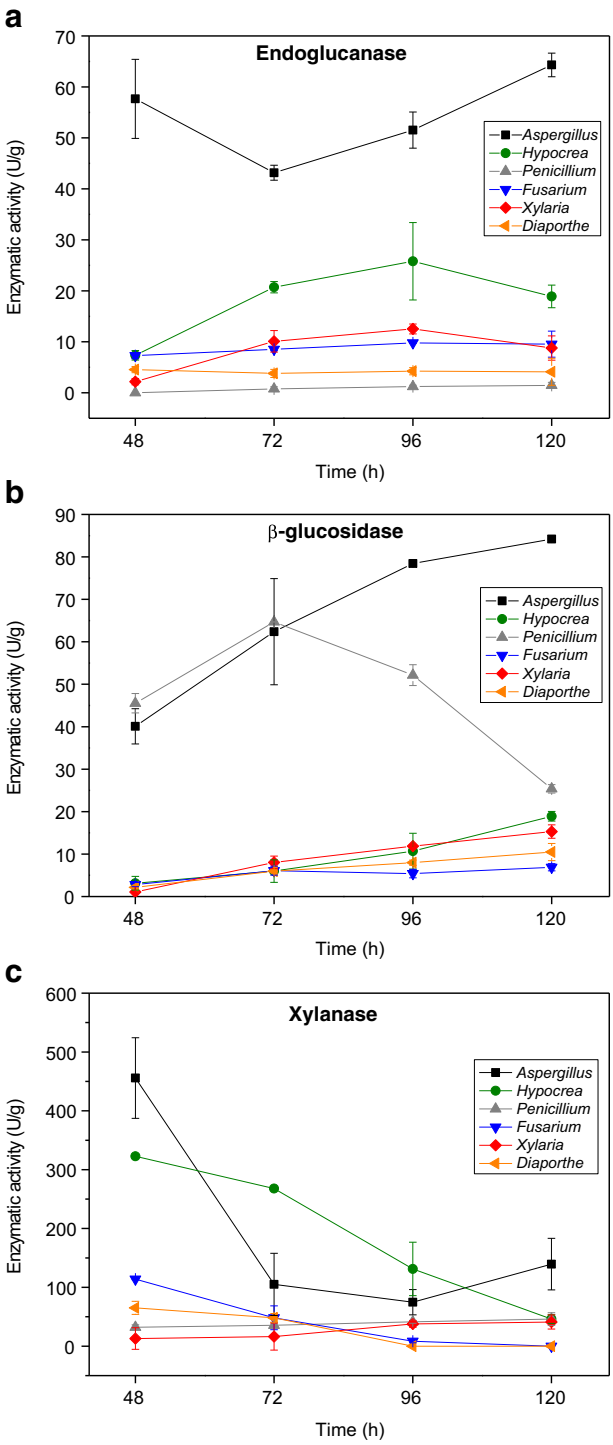

Fig. 2 Time profile of endoglucanase (**a**), xylanase (**b**), and β-glucosidase (**c**) enzyme production by the selected strains of the genus *Aspergillus*, *Hypocrea*, *Penicillium*, *Fusarium*, *Xylaria*, and *Diaporthe* cultivated under SSF was evaluated for up to 120 h

three genera are less studied in terms of the enzymatic potential in comparison to the genus *Aspergillus*, *Penicillium*, and *Trichoderma*.

Cultivations of Endophytic Fungi Strains Under SSF

After the selection of an estimated minimum SSF cultivation period for each of the six genus (*Aspergillus*, *Hypocrea*, *Penicillium*, *Fusarium*, *Xylaria*, and *Diaporthe*) that would be adequate for enzymatic production, the other strains were cultivated under SSF with samples analyzed at each of selected incubation time (Fig. 3). For this set of SSF cultivations, the strains *Aspergillus niger* F12 and *Trichoderma reesei* Rut-C30 were used as reference strains for (hemi)cellulolytic enzyme production.

In terms of endoglucanase enzymes produced by the endophytic strains (Fig. 3a), the strains of the genus *Aspergillus* achieved the highest values, with endoglucanase activity up to 63 U/g for the strain *A. awamori* 82 (4). This value was comparable to the endoglucanase production by the reference *A. niger* strain and even superior to the *T. reesei* Rut-C30. The *Diaporthe* 30 (3) strain also showed a significant endoglucanase activity (34 U/g), followed by most of the *Hypocrea* strains.

The production of β-glucosidase by the endophytic strains was especially higher for the *Penicillium* strains (up to 65 U/g) followed by the *Aspergillus* strains (up to 47 U/g) (Fig. 3b). In contrast, all the other endophytic genera produced relatively low amounts of β-glucosidase enzymes. The production of β-glucosidase by the endophytic *Penicillium* 60 (4) strain was even superior to the one achieved by the reference *A. niger* (49 U/g) that is recognized as a good producer of β-glucosidase enzymes [34, 44].

In terms of the production of xylanase enzymes by the endophytic strains (Fig. 3c), the highest activity values were obtained by the genera *Diaporthe*, *Aspergillus*, and *Hypocrea*. The strain *Diaporthe* 94 (4) stood out considerably in relation to its xylanase activity (77 U/g), a value higher than the reference *Aspergillus* and *Trichoderma* strains. This result indicates that this endophytic strain could be considered as a potential producer of xylanase enzymes.

It is important to highlight that the SSF cultivation conditions were still not optimized in the present study, suggesting that the values found for enzymatic production could be further improved. The characterization of each particular microorganism in terms of the influence of operational and environmental conditions such as source of nutrients, moisture content, and temperature on the kinetics of growth and product formation is essential for SSF bioprocess development [21, 45, 46]. For instance, a reduction of the moisture content of wheat bran from 70 to 50% resulted in a 2.5-fold improvement in endoglucanase production by a strain of *A. niger* (P47C3) isolated from the Amazon rainforest [47].

Enzymatic Hydrolysis of Cellulose

Eight strains displaying significant enzymatic activity values in the previous screening steps were selected for further investigation in terms of their performance on the hydrolysis of cellulose (Fig. 4). The hydrolytic potential of the SSF enzymatic cocktails of the endophytic strains of the genus *Aspergillus* 56 (3), 82 (4), and 108 (4); *Penicillium* 60(3) and 24(4); *Hypocrea* 1.16 and 48(4); and *Diaporthe* 94(4) was compared to that of the reference strains *A. niger* F12 and *T. reesei* Rut-C30.

The analysis of the cellulose hydrolysis experiments in terms of reducing sugars and glucose released as well as in terms of the conversion revealed that the *Aspergillus* 56 (3)

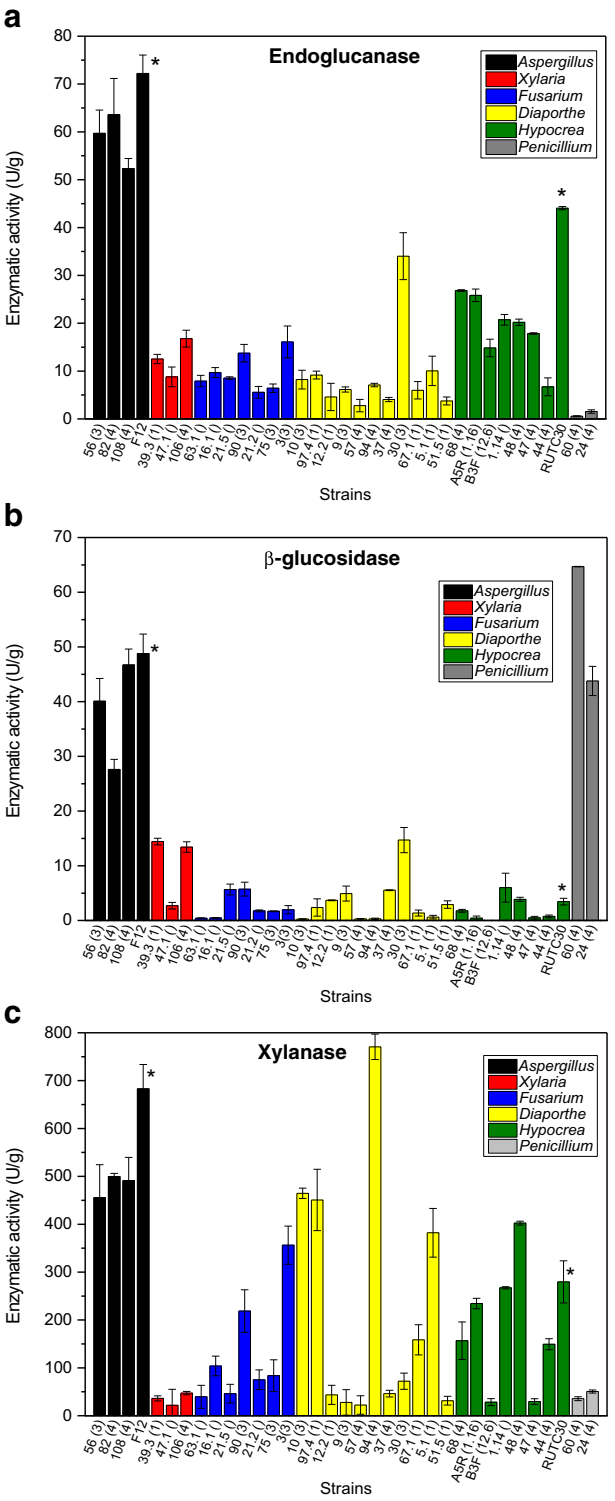

Fig. 3 Endoglucanase (**a**), xylanase (**b**), and β-glucosidase (**c**) enzymatic activity produced by the endophytic strains of the genus *Aspergillus*, *Hypocrea*, *Penicillium*, *Fusarium*, *Xylaria*, and *Diaporthe* cultivated under SSF. The reference strains *A. niger* F12 and *T. reesei* Rut-C30 are indicated by an asterisk

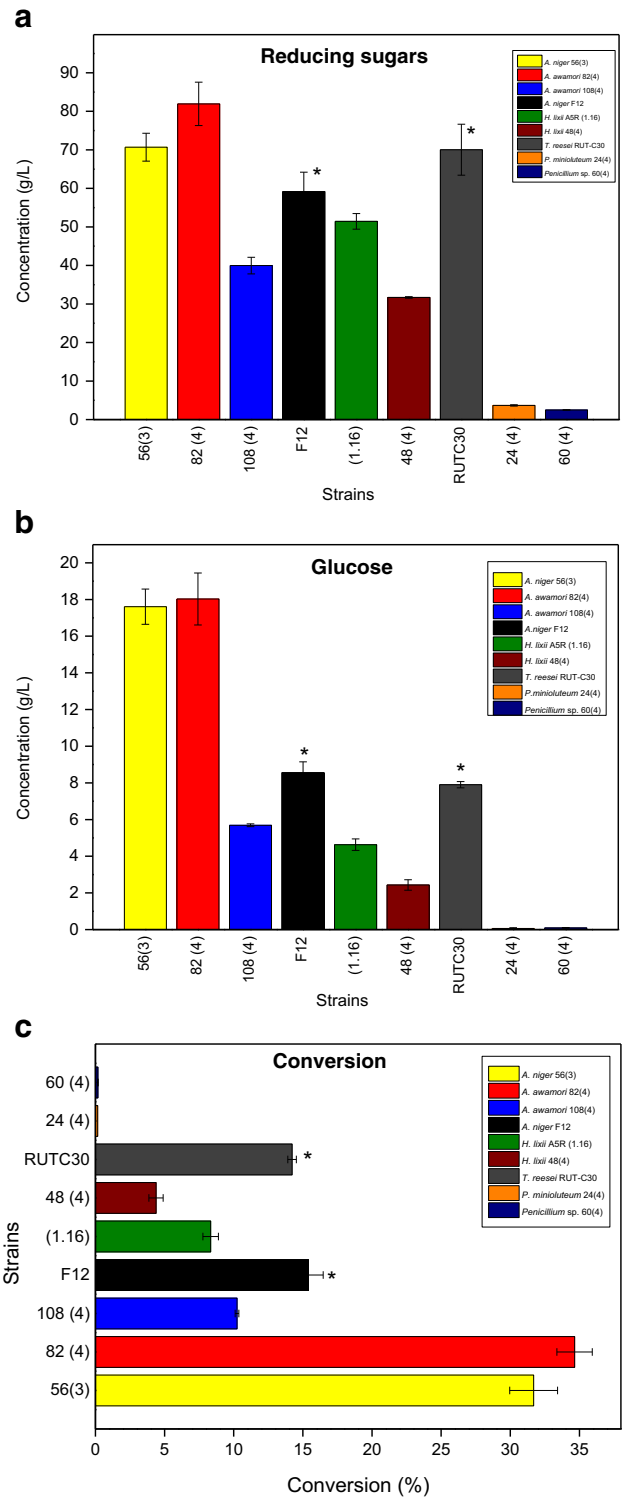

Fig. 4 The hydrolytic potential of the SSF enzymatic cocktails of the endophytic strains in terms of glucose release (**a**), total reducing sugars (**b**), and cellulose conversion (**c**) after 24 of reaction using 5% (*w*/*v*) solids loading of cellulose. The reference strains *A. niger* F12 and *T. reesei* Rut-C30 are indicated by an asterisk

and 82 (4) had a very distinguished performance, reaching cellulose conversion values of about 35% after 24 h (Fig. 4c). These values represent a more than twofold increase in comparison to the performance achieved using the enzymatic extracts from the reference strains of *Aspergillus* and *Trichoderma*. Besides, a comparison with literature values in terms of glucose released during the conversion of a cellulosic material showed that the glucose concentration released (up to 18 g/L) represents about tenfold the value previously reported for the cellulose conversion using in-house-produced enzymatic cocktails [48, 49].

The higher values of glucose released by using the enzymatic cocktails from the endophytic strains *Aspergillus* 56 (3) and 82 (4) give an indication that the β-glucosidase enzymes produced by these strains are more efficient and/or present a higher stability than the β-glucosidase produced by the reference *A. niger* strain. The *A. niger* used here as reference has been characterized in other studies as a good producer of β-glucosidase [34, 44]. However, it has been previously reported that there is a variety of β-glucosidase enzymes, from different families and which display different efficiencies towards the hydrolysis reaction, substrate inhibition, and stabilities as well [50]. For instance, some β-glucosidase belonging to the GH1 family exhibit unusual properties, being tolerant to or even stimulated by high glucose concentrations [51].

Interestingly, despite the inferior values of enzymatic activity presented by the endophytic strains *Aspergillus* 56 (3) and 82 (4) in comparison to the reference strain *A. niger* (Fig. 3), their performance on the cellulose hydrolysis was much superior (Fig. 4). A possible explanation for these results is related to the large amount of other important accessory enzymes involved in the hydrolysis of cellulosic materials [16, 22]. Therefore, such results highlight the potential of the enzymatic cocktails of the mangrove-associated endophytic strains *Aspergillus* 56 (3) and 82 (4) for the hydrolysis of biomass. Moreover, such endophytic strains showed a higher performance also when compared to the hyper-cellulolytic mutant *T. reesei* Rut-C30.

The natural adaptation of mangrove-associated endophytic fungi to the extreme environment of the mangrove ecosystem [31, 33] may have contributed to the development of such remarkable enzymatic arsenal. This observation is supported by the fact that the endophytic fungi are recognized as enzyme producers for their own natural needs, since a series of enzymes are necessary for penetrating and colonizing their plant hosts, including hydrolytic and oxidative enzymes [30, 52]. The extreme conditions of the mangrove ecosystem which comprises high salinity, tidal flooding, strong winds, and high temperatures [28, 31] may also have contributed to induce the production of enzymes with distinct characteristics.

In overall, linking hydrolysis performance to the screening steps showed to be a crucial step towards finding potential fungal strains for producing enzymatic cocktails with high efficiency in the saccharification of vegetal biomass. Such results can potentially contribute to the development of optimized enzymatic cocktails, thus reducing the enzyme loading and production cost of lignocellulosic biofuels by the biochemical pathway. Moreover, the potential of these mangrove-associated endophytic strains could be further explored for the production of enzymes on-site, within the biorefinery facilities.

Conclusions

The mangrove-associated endophytic fungi represent a potential source of microorganisms for industrial enzyme production. Here, the performance of the enzymatic cocktails from the selected endophytic strains on the hydrolysis of a cellulosic material revealed that the enzymes

from the endophytic strains of the *Aspergillus* genus resulted in conversion values more than twofold higher than the values achieved from reference strains of *Aspergillus* and *Trichoderma*. The natural adaptation of mangrove-associated endophytic fungi to the extreme environment of the mangrove ecosystem may have contributed to the development of such remarkable enzymatic arsenal. These results indicate the great potential of mangrove endophytic fungi for the production of carbohydrolases with efficient performance in the hydrolysis of biomass.

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Compliance with Ethical Standards

Competing Interests The authors declare they have no competing interests.

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