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Fucoidan-Degrading Fungal Strains: Screening, Morphometric Evaluation, and Influence of Medium Composition

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Abstract Ten different fungal strains from the genus *Aspergillus*, *Penicillium*, and *Mucor* were screened for fucoidan hydrolyzing ability aiming to find microorganisms able to produce sulfated fucan-degrading enzymes. Screening was carried out by measuring the strains kinetic and morphometric behavior over plate assays using *Laminaria japonica* fucoidan as only carbon source, testing three nitrogen sources (urea, peptone, and sodium nitrate). The selected fungal strains were subsequently used in submerged fermentations, which were performed for (1) selection of the strains able to growth over fucoidan medium and (2) media selection, testing the synergy of fucoidan with other sugars for inducing high enzyme titles. Radial expansion and hyphae parameters were observed for *Aspergillus niger* PSH, *Mucor* sp. 3P, and *Penicillium purpurogenum* GH2 grown only over fucoidan-urea medium. *A. niger* PSH showed the maximum enzymatic activity values, which were significantly different ($p<0.05$) from those achieved by the other selected fungi. Sucrose addition to fucoidan media proportioned the highest fucoidanase activity values for this fungal strain. This research allowed establishing optimal conditions for metabolites synthesis by fungal stains able to act toward fucoidan ramified matrix.

Keywords Fucoidan · Fucoidanases · Fungal strains · Screening · Fermentation

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Introduction

Fucoidan is a polysaccharide widely found in brown algae cell walls. This compound has a wide variety of biological activities including anticoagulant, antithrombotic, antitumoral, and antiviral being the most relevant against hepatitis, herpes, and human immunodeficiency (AIDS) viruses. The biological activity of fucoidans is determined by their specific chemical structure, which consist of a family of homo- and heteropolysaccharides composed mainly of fucose residues sulfated at positions 2 and/or 4 and bound by a-1,2- or a-1,3-*O*-glycosidic bonds. In addition to fucose, fucoidans may also contain mannose, xylose, galactose, and rhamnose sugars and uronic acids. Although the scientific and practical interest of studying the relation between structure and biology activity has been increased, a detailed elucidation of fucoidan chemical structure remains unknown [1–3].

Specific glycosyl hydrolase enzymes able to degrade fucoidan sulfated matrix are important tools to establish structural characteristics and biological functions of this polysaccharide. The literature available data mainly concern the fucoidanases of marine invertebrates such as *Haliotis* sp. and *Mizuhopecten yessoensis* [4, 5], but the main reports of endo- and exo- fucoidanases have been found in marine bacteria as *Vibrio* sp., *Pseudoalteromonas citrea*, *Pseudomonas* sp., *Alteromonas* sp., and *Flavobacteriaceae* sp., [6–8]. Information on terrestrial and fungal microorganisms acting over this sulfated-polysaccharide is scarce. Therefore, the research for microorganisms with high fucoidan hydrolase activity remains a challenge.

Following the principle that polysaccharide degradation can be determined by monitoring microbial growth on plate media with a specific carbon source, polysaccharidase-producing microorganisms can be found from enrichment cultures on the target polysaccharide. Studies on image analyses of fungal strains have helped to relate morphological features to enzymes production aspects (pectinase and amylase) and the influence of culture media on growth patterns [9–11]. Fungal filamentous growth can be interpreted on the basis of a regular cell cycle; therefore, mycelial growth and morphology can be mathematically described by validated kinetic models for the estimation of specific growth rate (μ) of molds on agar plates by image processing techniques [12, 13].

The aim of this study was to identify fungal strains able to growth over fucoidan-based media and produce active fucoidanases. Specifically, the quantification of kinetic and morphology features over agar plate aiming to establish the influence of media composition on growth patterns was investigated. The interaction between different saline media and carbon sources as enzyme inducers in submerged fermentations was also evaluated.

Materials and Methods

Reagents

Fucoidan of *Laminaria japonica* was purchased from Rizhao Jiejing Ocean Biotechnology Development Co., Ltd. Coomassie Plus (Bradford) Assay Kit were from Thermo Scientific Co. Anthrone reagents were from Prolabo, Normapur, Merck and 3,5-dinitrosalicylic acid from Fluka, Chemika. Other reagents were all of analytical grade.

Fungal Strains and Inoculum Preparation

Ten filamentous fungi isolated from northeast Mexican desert, which belong to the collection of the Food Research Department (DIA-UAC) from the University of Coahuila

(Saltillo, Mexico), reported as microorganisms previously used for the induction of nonconstitutive enzymes [14], were used in the present work. The strains were identified in the Micoteca of the University of Minho (MUM, Braga, Portugal), being classified as five *Aspergillus* (*Aspergillus ustus* PSS, *Aspergillus niger* ESH, *A. niger* GH1, *A. niger* PSH, and *A. niger* AA20), four *Penicillium* (*Penicillium pinophilum* EH2, *P. pinophilum* EH3, *Penicillium* sp. ESS, and *Penicillium purpurogenum* GH2), and one *Mucor* sp. (3P). For inoculum preparation, fungal spores were transferred to potato dextrose agar medium and incubated at 30 °C for 7 days. Spores were scraped into 0.01% (*v*/*v*) Tween 80 solution and counted in a Neubauer chamber to be inoculated in the fermentation media.

Culture Media and Fermentation Conditions

Screening of Fungal Strains

Different culture media containing fucoidan as sole carbon source were used for the fungal strains screening. Culture media minimal medium (MM) were composed by (grams per liter): fucoidan (5.0), nitrogen source (2.0), and agarose (10.0), dissolved in 100 mM acetate buffer (pH 4.5). Urea, peptone, and sodium nitrate were tested as nitrogen sources. For comparative experiments, culture media CZ, containing Czapek dox mineral salts, were used. Such media were composed by same carbon and nitrogen sources of media MM plus the addition of mineral salts in the following concentrations (grams per liter): KH_2PO_4 (1.0), $MgSO_4$ (0.5), and KCl (0.5). Culture media containing only nitrogen sources were used as growth blank.

After preparation, the media were poured on 60-mm Petri plates and inoculated in the center with a spore suspension containing 1×10^6 conidia mL^{-1}. The plates were statically incubated at 30 °C during 5 days. Cultivations were done in triplicate to each fungal strain in each different culture media.

Fermentation Conditions for Microorganism Selection

In this stage, the fermentation assays were carried out in 250-mL Erlenmeyer flasks containing 50 mL of culture media MM composed by fucoidan and urea, 10.0 and 5.0 g L^{-1}, respectively. Culture media had their pH adjusted to 5 by addition of HCl 0.5 M solution and were sterilized by microfiltration (membrane filters of 0.2 μm, Millipore). The flasks were inoculated with 1×10^6 spores mL^{-1} and maintained at 30 °C, 140 rpm, during 5 days. Cultivations were done in duplicate to each fungal strain selected from the previous stage.

Fermentation Conditions for Media Selection

These assays were performed for evaluation of the interaction effect between fucoidan (primary carbon source) and a secondary carbon source: glucose, sucrose, lactose, fructose, or sodium acetate. The fermentation media were composed by (grams per liter): fucoidan (10.0), secondary carbon source (5.0), and urea as nitrogen source (5.0), supplemented with Czapek Dox (CZ) or Pontecorvo (PC) mineral salts. For CZ media, the mineral salts and their respective concentrations were the same described in the "Screening of Fungal Strains". For PC media, the mineral salts and their concentrations were (grams per liter): KH_2PO_4 (1.5), $MgSO_4$ (0.5), KCl (0.5), $FeSO_4\times7H_2O$ (0.001), $ZnSO_4\times7H_2O$ (0.001), $MnCl_2\times4H_2O$ (0.001), and $CuSO_4\times5H_2O$ (0.001).

The fermentations assays were carried out in 250-mL Erlenmeyer flasks containing 50 mL of culture media (pH adjusted to 5 by addition of HCl 0.5 M) sterilized by microfiltration (membrane filters of 0.2 μm, Millipore). The flasks were inoculated with 1×10^6 spores mL^{-1} and maintained at 30 °C, 140 rpm, during 5 days. Cultivations were done in duplicate only for the previous selected fungal strain.

Analytical Methods

Sugars Consumption

Culture media consumption was quantified as the intake percentage of total sugars (TS), which were quantified by anthrone method [15] using glucose as standard.

Biomass and Radial Growth Rate

Fungal biomass during the submerged fermentations was evaluated by dry weight, where the mycelium obtained after filtration (membrane filter 0.45 μm, 47 mm) was dried at 150 W for 20 min. Radial growth rate (U_r) was kinetically monitored measuring colony diameters every 24 h for 5 days, using a digital micrometer (Mitutoyo 293-561, Japan). Slopes of radial growth were estimated by linear regression (R^2=0.95) and expressed as micrometers per hour. Final colonies were saved for being observed on the microscope in order to obtain average data for hyphal length and diameter to each fungal strain. Specific growth rates (μ, per hour) were calculated according to the following model [13]:

$$\mu = \frac{U_r \ln(2)}{L_{av} \ln\left(\frac{L_{av}}{D_h}\right)} \quad (1)$$

where U_r was the radial growth rate (micrometers per hour), L_{av} was the average length of leading hyphae in the periphery of the colonies (micrometers), and D_h was the mean diameter of hyphal tubules (micrometers).

Protein and Enzymatic Activity

Total protein concentration in fungal extracts was determined with a Bradford protein assay kit (Bradford reagent, Pierce). Enzymatic activity was measured by the dinitrosalicylic acid technique, which estimates the reducing sugars released during the reaction between 900 μL of substrate (fucoidan 10 g L^{-1} in 200 mM acetate buffer, pH 4.5) and 100 μL of enzyme extract, at 37 °C for 24 h. One unity (U) of enzyme was defined as the amount of enzyme able to release 1 μmol of reducing sugars per hour. The data correspond to triplicates of independent experiments.

Image Acquisition

For image analyses measurements, a digital camera AxioCam HRc (Zeiss, Oberkochen) mounted on a Zeiss Axioskop microscope (Zeiss, Oberkochen) was used. Phase contrast with ×10 and ×40 objective lenses were selected for hyphal length (L_{av}) and diameter (D_h) measurements, respectively. Three plates of each fungus in each different medium were analyzed. The observation fields were selected randomly along the entire periphery plate, and samples of 30 hyphal tubes per plate were quantified. The obtained images were

analyzed in an Axiovision Software version 4.7. Mean values were calculated by a frequency distribution.

Calculation of Metabolic Parameters

The metabolic parameters including specific growth rate (μ), biomass yield ($Y_{X/S}$), enzyme yield ($Y_{E/X}$), and enzyme productivity (P_R) were calculated. Specific growth rate was obtained from growth curves fitted by Microsoft Excel's Solver tool, using the logistic equation. The biomass yield was defined as the amount of biomass produced per gram of sugar consumed, the enzyme yield as the enzyme activity per gram of biomass, and the enzyme productivity as the rate of enzyme synthesis per liter per hour.

Statistical Analyses

The difference among samples was verified by using the Tukey's range test. A p value of less than 0.05 was regarded as significantly different. Statgraphics Plus for Windows version 4.1 was the software used for data analysis.

Results and Discussion

Strains Selection on Petri Plates

Growth fungal evaluation over fucoidan-based media was carried out with the purpose of find terrestrial microorganisms able to degrade this complex polysaccharide. From the ten strains assessed in the three different nitrogen sources, only *A. niger* PSH, *P. purpurogenum* GH2, and *Mucor* sp. showed mycelium presence over fucoidan-urea plates in MM medium after 24 h of inoculation. Among these three selected strains, *Mucor* sp. 3P and *A. niger* PSH colonies completely covered the agar plate after 6 days of incubation while *P. purpurogenum* GH2 required more than 10 days of cultivation to invade half of Petri plate.

On the contrary of urea medium, the culture media containing other nitrogen sources (peptone or sodium nitrate) did not show any strain growth. Fungal colonies formation over urea could have been influenced by the induction of urease, which could have promoted the extracellular fucoidanase production. The urease enzyme is usually constitutive in fungal mycelia and is able to degrade urea to ammonium and CO_2. Ammonium ions are readily translocated and can be assimilated into glutamate and glutamine, which serve as precursors for the biosynthesis of many important cellular macromolecules [16, 17]. In addition, urea appears to be the major nitrogen source in the sea, generally present at levels of 0.1–1 μM [18, 19]; therefore, it can be a natural promoter of the fucoidan hydrolytic enzyme.

Hyphal Growth Measurements

The image analysis measurements of hyphal length and diameter (Fig. 1) were useful to classify fungal strains tested in selective culture media. Table 1 shows the morphological parameters obtained by fungal cultivation on fucoidan-urea Petri plates, comparing MM and CZ media. Differences among the fungal strains and media were perceptible, but all the strains showed a direct relation between the kinetic (U_r) and micrometrical parameters (L_{av} and D_h).

For each evaluated medium, the highest parameter values were always obtained by cultivation of the *Mucor* 3P strain, while *P. purpurogenum* GH2 gave the lowest results.

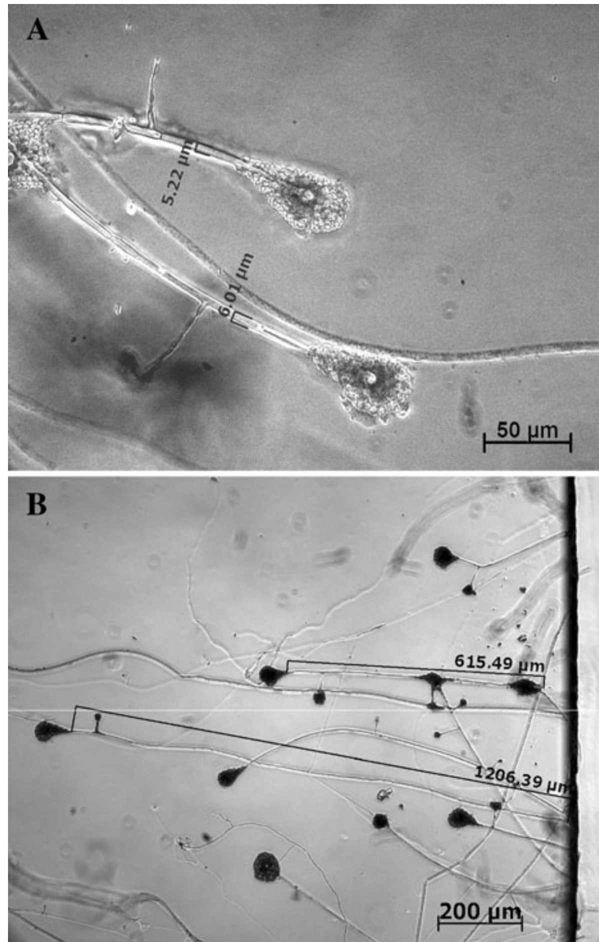

Fig. 1 Image analysis measurement on contrast phase photography of *Mucor* sp. 3P growth on fucoidan-urea minimal medium (MM). **a** Hyphae diameter, magnification ×40, and **b** hyphae length, magnification ×10

Microscopic observation of hyphal diameters (D_h) did not show significant differences among the fungal strains grown on MM. On the other hand, the hyphal length (L_{av}) of *Mucor* 3P had an average value 36% higher than that measured for *A. niger* PSH, which can be expressed as a major degree of branching. Mineral salts addition to the media (CZ)

Table 1 Growth parameters of fungal strains cultivated on fucoidan-urea Petri plates.

Cultivation media	Strain	U_r (µm h^{-1})	L_{av} (µm)	D_h (µm)	μ (h^{-1})
Minimal (MM)	*Mucor* 3P	579.90±0.01	251.63±89.21	4.44±0.89	0.40
	A. niger PSH	350.43±0.03	184.47±48.18	4.41±0.77	0.37
	P. purpurogenum GH2	136.93±0.09	158.99±66.07	3.40±1.17	0.16
Czapek (CZ)	*Mucor* 3P	755.07±0.01	336.68±107.69	7.05±1.72	0.40
	A. niger PSH	390.67±0.01	208.33±61.77	5.37±1.06	0.36
	P. purpurogenum GH2	232.80±0.16	206.29±53.09	3.51±0.80	0.19

U_r radial growth rate; maximal rate of extension of leading hyphae, L_{av} average length of hyphae in the periphery of the colonies, D_h mean diameter of hyphal tubules, μ specific growth rate

enhanced germination and growth of germ tube, allowing higher conidial elongation (L_{av}). On average, when cultivated in CZ media, *Mucor* 3P, *A. niger* PSH, and *P. purpurogenum* GH2 presented elongation rates 33%, 12%, and 29% higher than those obtained in MM media; however, hyphal diameter was only significantly influenced on *Mucor* 3P. A possible explanation for this is that length and diameter are not regulated at the same physiological level during the growth process since the hyphae diameter is defined at the beginning of the process, while the hyphae length remains elongating for a longer time. In addition, it was also found that elongation and diameter of the hyphae are closely related to the size of the spores, varying thus for the different fungal species [20]. Therefore, the large spores of *Mucor* sp. provided fast growing of thick hyphae, whereas the small spores of *Aspergillus* and *Penicillium* species produced slow growing of thin hyphae.

Among the three evaluated fungal strains, *Mucor* sp. 3P gave the highest specific growth rate (μ) values (Table 1). Similar results were obtained by cultivation of *A. niger* PSH in both media (MM and CZ), while *P. purpurogenum* GH2 provided the lowest results. Besides the highest specific growth rate, *Mucor* 3P gave also the highest maximal rate of extension of leading hyphal (U_r, micrometers per hour), mainly when CZ medium was used.

The specific growth rate (μ, per hour) is a valid physiological parameter for comparing fungal growth patterns measuring mycelial branching frequencies in order to estimate biomass formation. Cumulative percentage profiles of the total measured hyphae in CZ and MM media were then plotted against the hyphae length and diameter (Fig. 2). High polydispersity was observed for the three fungi, as an evidence of the heterogeneous hyphae tips, which can be a response of metabolic stress at the induced media composition. No differences on diameter profile in *A. niger* PSH and *Mucor* 3P in MM media were observed (Fig. 2b), and thus, medium assimilation was only differed by the length profile (Fig. 2a). The presence of fungal colonies evidence that such microorganisms are able to excrete metabolites with hydrolytic action over the fucoidan branched structure.

Production of Fucoidan-Degrading Enzymes by Selected Strains

Submerged fermentation experiments were carried out for the strains that showed capacity to grow over fucoidan plates (*A. niger* PSH, *P. purpurogenum* GH2, and *Mucor* sp. 3P). During their cultivation under submerged fermentation conditions, they presented dispersed mycelial filaments, a particular morphological form that can be resulted from the nature of the inoculum as well as the minimal medium composition used in the experiments [21].

Concerning to biomass production, the highest results were obtained with *Mucor* sp. 3P and *A. niger* PSH (0.849 and 0.464 g L^{-1}, respectively) after 48 h cultivation, time after which both strains reached the stationary phase (Fig. 3a). On the contrary, as previously observed on agar plate experiments, *P. purpurogenum* GH2 showed the lowest biomass growth, reaching values closed to 0.274 g L^{-1}. Statistical analysis of these data (Table 2) only revealed differences significant at 95% confidence level between *Mucor* sp. 3P and *P. purpurogenum* GH2. The maximum substrate consumption obtained with *Mucor* sp. 3P was also statistically different of those obtained with the other fungal strains. By contrast, the enzyme activity was the highest for *A. niger* PSH, with values oscillating from 0.441 to 0.613 U L^{-1} after 48 h of fermentation (Fig. 3b), which were significantly different ($p<0.05$) of those obtained for *P. purpurogenum* GH2 and *Mucor* sp. 3P. In brief, *Mucor* sp. 3P was the strain with highest ability to growth and consume substrate; otherwise, *A. niger* PSH showed the highest enzymatic activity and its biomass production was not statistically different from the results achieved by *Mucor* sp. 3P. Based on these results, *A. niger* PSH was the fungal strain selected for use in the subsequent step.

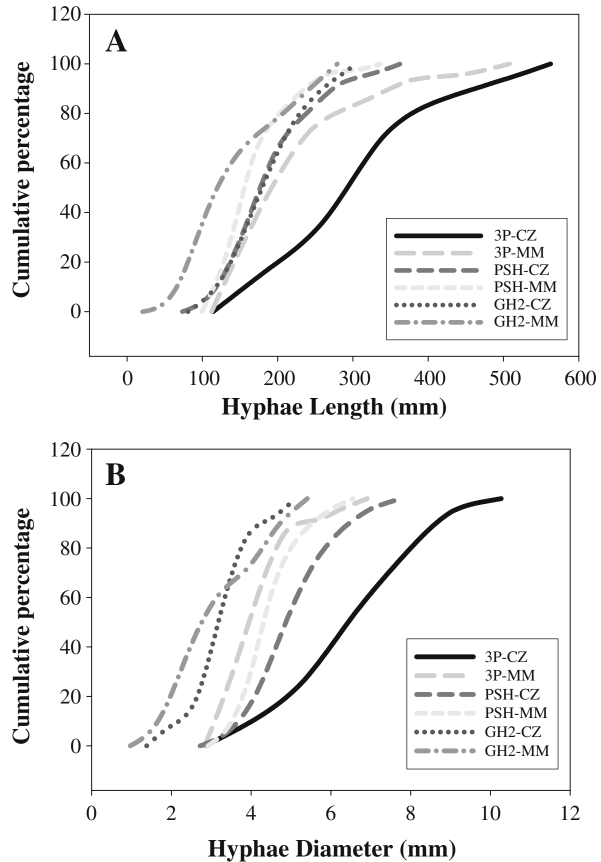

Fig. 2 Cumulative percentage profile of hyphae length (**a**) and diameter (**b**) during the fungi cultivation in minimal (MM) and Czapek (CZ) media. *3P Mucor* sp., *PSH A. niger*, *GH2 P. purpurogenum*

Effect of Combined Media on Biomass Growth, Substrate Consumption, and Induction of Fucoidan-Degrading Enzymes

The biomass results obtained during the *A. niger* PSH cultivation in the combined media are shown in Fig. 4. Note that the maximum biomass production (5.79 g L^{-1}) was reached on Czapek medium containing fucoidan supplemented with sucrose, but the strain also grew well on the culture media supplemented with glucose or fructose (3.83 and 3.10 g L^{-1}, respectively). On the contrary, low mycelia production was observed when using fucoidan as sole carbon source or supplemented with lactose or sodium acetate as secondary carbon source, being not observed significant differences ($p<0.05$) among these three assays (Table 3).

Regarding the substrate consumption, the best results once more were obtained in media supplemented with sucrose, glucose, or fructose (Table 3). However, in this case, the highest intake (79.93% of total sugars) was obtained in glucose supplemented medium. Sodium acetate addition did not promote significant differences from the medium containing fucoidan, but the addition of lactose favored the sugars consumption compared with this medium, although such consumption did not reflect in a highest biomass production. Otherwise, sucrose supplemented medium showed lower substrate consumption than that obtained in glucose supplemented medium, which means that sucrose

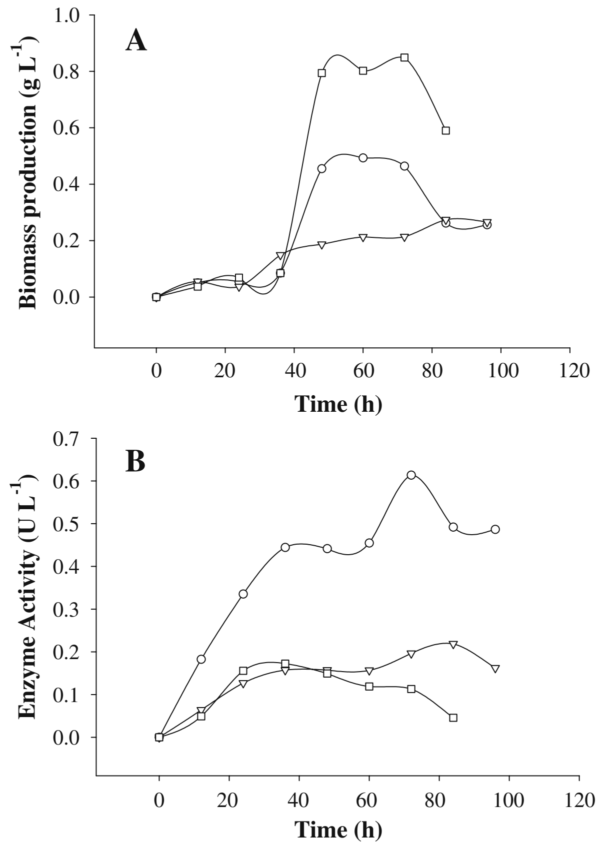

Fig. 3 Submerged fermentation over fucoidan-urea medium of *A. niger* PSH (*circle*), *Mucor* sp., (*square*), and *P. purpurogenum* GH2 (*inverted triangle*). **a** Biomass production; **b** enzyme activity

supplementation allowed a higher cellular growth per substrate consumed, as can be confirmed by the $Y_{X/S}$ values given in Table 4.

Fucoidan hydrolytic enzymes were only expressed as extracellular metabolites in all the evaluated Czapek media supplemented with a secondary carbon source, being not detected any intracellular activity for all the studied media. Enzyme activity was the highest in the sucrose supplemented medium, whose value (2.77 U L^{-1}) was significantly different ($p<0.05$) of that attained in the medium containing fucoidan as sole carbon source (1.88 U L^{-1}; Table 3). Kinetic profiles of fucoidanase activity are shown in Fig. 5, which reveals a constant behavior between 48–96 and 72–96 h of fermentation for sucrose-supplemented

Table 2 Multiple comparison procedure to discriminate among the biomass, substrate consumption, and enzymatic activity means obtained during the submerged fermentation for microorganism selection.

Strain	Biomass (g L^{-1})	Substrate (g/100 g TS)	Enzyme activity (U L^{-1})$^{\alpha}$
Mucor sp. 3P	0.85 b	17.17 b	0.17 a
A. niger PSH	0.46 ab	9.76 a	0.61 b
P. purpurogenum GH2	0.27 a	11.88 a	0.22 a

Different letters mean values statistically different at 95% confidence level

$^{\alpha}$U L^{-1} =(μmol/h)/L

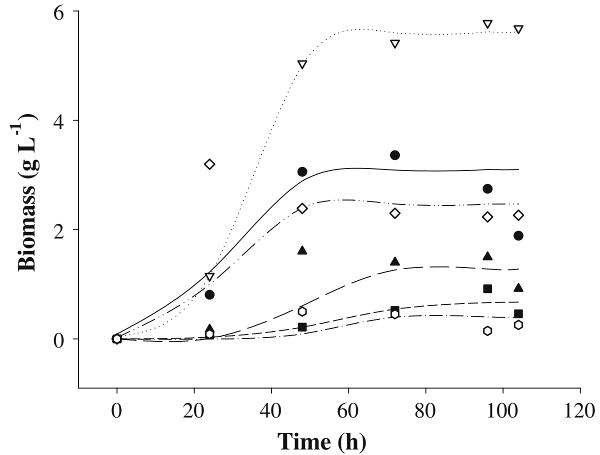

Fig. 4 Biomass production of *A. niger* PSH during the submerged fermentation in Czapek medium containing fucoidan supplemented with: glucose (●, —), sucrose (▿, ····), lactose (■, - - -), fructose (◇, -···-), or sodium acetate (▲, − −). Medium containing fucoidan as sole carbon source (○, -···-). *Symbols* are experimental data; *lines* are calculated data adjusted by logistic equation (R^2>0.90)

and fucoidan media, respectively; however, after 96 h, the activities expression decreased abruptly in both media. Curiously, only any fucoidanase activity was detected in fructose supplemented medium, suggesting that the good results of biomass production and substrate consumption observed above could be related to the production of other proteins. As can be seen in Table 3, total protein production was the highest in media supplemented with sucrose or fructose (22.80 and 16.03 mg L^{-1}, respectively), while the lowest protein values were found for the medium supplemented with sodium acetate, which did not show significant differences from the medium containing only fucoidan.

The kinetic parameters obtained during the fungal fucoidanase production by liquid fermentation using different culture media are shown in Table 4. Confirming the above discussed idea, the highest biomass yield ($Y_{X/S}$=0.87 g g^{-1}) was obtained in Czapek medium containing fucoidan supplemented with sucrose. On the other hand, the highest enzyme yields ($Y_{E/X}$=3.55 and 1.01 U g^{-1}) were achieved in the media containing fucoidan as sole carbon source or supplemented with lactose, which can be explained by the low biomass formation attained in these media. Enzyme productivity values (P_R) showed that sucrose supplemented medium allowed the synthesis of fucoidan-degrading enzymes in a rate similar to that obtained in the medium containing only fucoidan as carbon source.

Table 3 Multiple comparison procedure to discriminate among the biomass, substrate consumption, enzymatic activity, and protein means obtained during the submerged fermentation for media selection.

Culture media	Biomass (g L^{-1})	Substrate (g/100 g TS)	Enzyme activity (U L^{-1})$^{\alpha}$	Protein (mg L^{-1})
Glu/Fuc	3.84 b	79.93 d	0.67 b	15.03 b
Suc/Fuc	5.80 c	44.28 c	2.77 e	22.81 c
Fru/Fuc	3.11 b	53.38 c	0.00 a	16.00 bc
Lac/Fuc	0.92 a	28.84 b	0.93 c	4.83 a
Ac/Fuc	1.62 a	20.03 a	0.20 a	3.84 a
Fuc	0.53 a	8.52 a	1.88 d	4.81 a

Different letters means values statistically different at 95% confidence level

Glu glucose, *Suc* sucrose, *Fru* fructose, *Lac* lactose, *Ac* sodium acetate, *Fuc* fucoidan, *TS* total sugars

$^{\alpha}$U L^{-1} =(μmol/h)/L

Table 4 Kinetic parameters obtained during the fungal fucoidanase production by submerged fermentation using different culture media.

Culture media	$Y_{X/S}$	$Y_{E/X}$	P_R
Glu/Fuc	0.32	0.17	0.09
Suc/Fuc	0.87	0.48	0.40
Fru/Fuc	0.39	0.00	0.00
Lac/Fuc	0.21	1.02	0.08
Ac/Fuc	0.54	0.13	0.04
Fuc	0.62	3.55	0.50

$Y_{X/S}$=g biomass per g substrate, $Y_{E/X}$=U per g biomass, P_R=U L^{-1} h^{-1}

Glu glucose, *Suc* sucrose, *Fru* fructose, *Lac* lactose, *Ac* sodium acetate, *Fuc* fucoidan

It is worth mentioning that, when Pontercorvo saline medium was used instead of Czapek, all the tested carbon sources also enabled the biomass formation, and likewise on Czapek medium, sucrose supplementation gave the highest values (4.77 g L^{-1}) followed by fructose and glucose supplementations (3.46 and 3.31 g L^{-1}, respectively). Lactose and sodium acetate supplemented media did not show significant differences from the lowest values obtained with fucoidan as sole carbon source. However, fucoidanase enzymatic activities were not detected in theses assays, suggesting that the synthesis of this enzyme could have been inhibited by the presence of microelements (Ca, Mg, Mn, and Cu) in the Pontecorvo formulation.

As a whole, the results obtained in the present work can be well compared with those reported for marine bacteria and invertebrates from other sources [1, 5, 22]. The differences of enzyme activity synthesis observed for the six evaluated media are probably due to the specificity of the enzymes production for some features of the substrate structure, combining simple sugar content and the complex fucoidan polysaccharide, with structural impediment such as the different position of sulfate groups and their sulfatation degree.

Conclusions

In conclusion, the use of agar plate quantitative method was an important tool for the establishment of preliminary physiological differences in fungal strains performance as

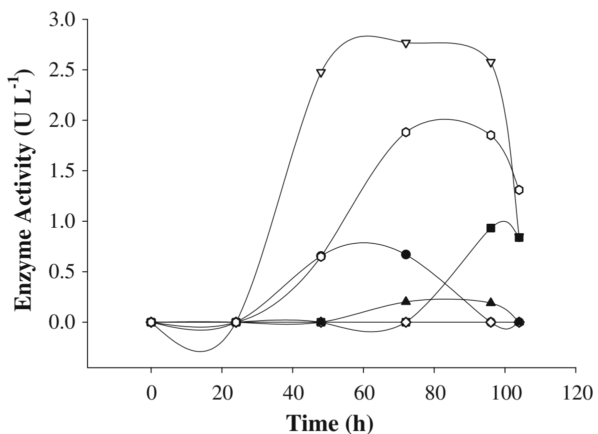

Fig. 5 Fucoidanase activity of *A. niger* PSH during the submerged fermentation in Czapek medium containing fucoidan supplemented with: glucose (*filled circle*), sucrose (*open inverted triangle*), lactose (*filled square*), fructose (*open diamond*), or sodium acetate (*filled triangle*). Medium containing fucoidan as sole carbon source (*open circle*)

enzymes producers. *A. niger* PSH, *P. purpurogenum* GH2, and *Mucor* sp. 3P are able to growth on different fucoidan-urea media; however, only *A. niger* showed great importance for the synthesis of sulfated fucan-degrading enzymes. Differences in the culture medium composition had a significant effect on the fungi growth, and the production of secondary metabolites was substantially affected. Sucrose supplemented medium proportioned the best results, while the sodium acetate addition did not show any significant improvement ($p<0.05$) in the results obtained from fucoidan as sole carbon source. These are the first results describing the production of enzymes from terrestrial fungus with ability to degrade fucoidan. In the next step, studies will be performed aiming to optimize the fermentative conditions in order to maximize the enzyme production.

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References

1. Alexeeva, Y. V., Ivanova, E. P., Bakunina, I. Y., Zvaygintseva, T. N., & Mikhailov, V. V. (2002). *Letters in Applied Microbiology, 35*, 343–346. doi:10.1046/j.1472-765X.2002.01189.x.
2. Ellouali, M., Boisson-Vidal, C., Durand, P., & Jozefonvicz, J. (1993). *Anticancer Research, 13*, 2011–2019.
3. McClure, M. O., Moore, J. P., Blanc, D. F., Scotting, P., Cook, G. M., Keynes, R. J., et al. (1992). *AIDS Research and Human Retroviruses, 8*, 19–26.
4. Sakai, T., Kawai, T., & Kato, I. (2004). *Marine Biotechnology, 6*, 335–346. doi:10.1007/s10126-003-0033-5.
5. Urvantseva, A. M., Bakunina, I. Y., Nedashkovskaya, O. I., Kim, S. B., & Zvyagintseva, T. N. (2006). *Applied Biochemistry and Microbiology, 42*, 484–491. doi:10.1134/S0003683806050073.
6. Bakunina, I. Y., Nedashkovskaya, O. I., Alekseeva, S. A., Ivanova, E. P., Romanenko, L. A., Gorshkova, N. M., et al. (2002). *Microbiology, 71*, 41–47. doi:10.1023/A:1017994131769.
7. Furukawa, S., Fijikawa, T., Koga, D., & Ide, A. (1992). *Bioscience, Biotechnology, and Biochemistry, 56*, 1829–1834.
8. Giordano, A., Andreotti, G., Tramice, A., & Trincone, A. (2006). *Biotechnology Journal, 1*, 511–530. doi:10.1002/biot.200500036.
9. Carlsen, M., Spohr, A., Nielsen, J., Villadsen, J. (1996). *Biotechnology and Bioengineering*, *49*, 266–276. doi:10.1002/(SICI)1097-0290(19960205)49:3<266::AID-BIT4>3.0.CO;2-I
10. Minjares-Carranco, A., Viniegra-Gonzalez, G., Trejo-Aguilar, B. A., & Aguilar, G. (1997). *Enzyme and Microbial Technology, 21*, 25–31. doi:10.1016/S0141-0229(96)00212-8.
11. Rodríguez-Gómez, D., Loera, O., Saucedo-Castañeda, G., & Viniegra-González, G. (2009). *World Journal of Microbiology & Biotechnology, 25*, 513–518. doi:10.1007/s11274-008-9917-x.
12. Larralde-Corona, C.P., Lopez-Isunza, F., & Viniegra-González, G. (1997) *Biotechnology and Bioengineering*, *56*, 287–294. doi:10.1002/(SICI)1097-0290(19971105)56:3<287
13. Loera, O., & Viniegra, G. (1998). *Biotechnology Techniques, 12*, 801–804. doi:10.1023/A:1008824903979.
14. Cruz-Hernandez, M., Contreras-Esquivel, J. C., Lara, F., Rodriguez, R., & Aguilar, C. N. (2005). *Zeitschrift fur Naturforschung, 60c*, 844–848.
15. Dreywood, R. (1946). *Industrial and Engineering Chemistry, Analytical Edition, 18*, 499. doi:10.1021/i560156a015.
16. Kavanagh, K. (2005). *Fungi: Biology and applications*. New York: Wiley.
17. Moure, D. (1998). *Fungal morphogenesis*. Cambridge: Cambridge University Press.
18. Antia, N. J., Harrison, P. J., & Oliveira, L. (1991). *Phycologia, 30*, 1–89.
19. Collier, J. L., Brahamsha, B., & Palenik, B. (1999). *Microbiology, 145*, 447–459. doi:10.1099/13500872-145-2-447.
20. Juniper, S., & Abbott, L. K. (2006). *Mycorrhiza, 16*, 371–379. doi:10.1007/s00572-006-0046-9.
21. Papagianni, M. (2004). *Biotechnology Advances, 22*, 189–259. doi:10.1016/j.biotechadv.2003.09.005.
22. Burtseva, Y. V., Kusikin, M. I., Sova, V. V., Shevchenko, N. M., Skobun, A. S., & Zvyagintseva, T. N. (2000). *Russian Journal of Marine Biology, 26*, 453–456. doi:10.1023/A:1009459107956.