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# Identification and evaluation of fungal strains with fucoidan degradation potential

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## Abstract

Ten fungal strains isolated of Mexican semi-desert were screened for ability to hydrolyze fucoidan in order to search microorganism capable to produce sulfated fucans-degrading enzymes. Plate assay and liquid fermentation experiments were carried out using *Laminaria japonica* fucoidan as only carbon source, testing three nitrogen sources. Growth was observed only in *Aspergillus niger* PSH, *Mucor sp*., and *Penicillum purpurogenum* GH2 in fucoidan-urea medium. The activity of fucoidanases was determined by reduced sugars. *Aspergillus niger* PSH showed the highest activity titles. This research indicate that filamentous fungi, using specific medium, are sources enable to induce active metabolism that act toward this class of polysaccharide.

## 1 Introduction

Fucoidan is a sulfated fucose hetero-polysaccharide, found in cell walls of brown algae with a wide variety of biological activities including anticoagulant, antithrombotic, antitumoral and antiviral being the most relevants against hepatitis, herpes and human immunodeficiency (AIDS) viruses (Berteau and Mulloy, 2003; Ellouali et al., 1993; McClure et al., 1992). Morever, preparations of polysaccharides with standard characteristics as well as medicines and supplements from these biopolymers also include enzymatic treatments. Although, scientific and practical interest of studying the relation between structure and biological activity has been studied, a detailed chemical structure of fucoidan elucidation remains unknown.

Specific (Glycosylhydrolases) enzymes capable to tailored fucoidan sulfated matrix are important tools for establish the structural characteristics and biological functions of this class of polysaccharide. Endo- and exo- fucoidanases have been principally found in marine bacteria as *Vibrio* sp. (Furucawa et al., 1992), *Pseudoalteromonas citrea* (Bakunina et al, 2002) *Pseudomonas sp.*, *Alteromonas sp.* (Sakai et al., 2004 ), *Flavobacteriaceae* sp., (Urvantseva et al., 2006), and marine invertebrates as *Haliotis* sp., *Mizuhopecten yessoensis* mollusks and *Strongylocentrotus nudus* sea urchin (Giordano et al., 2006).

There are any reports of terrestrial and neither fungal microorganism that hydrolyzes fucoidan. For that reason, the aim of the present work was to identify fungal strains that can growth over fucoidan medium as sources of active fucoidanases, and to evaluate enzymes excretion capacity over minimal conditions.

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## 2 Materials and methods

Fucoidan of *Laminaria japonica* (molecular weight 100,000 Da) was obtained from Rizhao Jiejing Ocean Biotechnology Development Co.,Ltd.

The filamentous fungus studied in the present work were isolated from Northeast Mexican desert (Cruz-Hernandez et al., 2005), collected during scientific expeditions realized by the Fermentation Group of Food Research Department, Universidad Autonoma de Coahuila, (Saltillo, Mexico). Strains identification was carried out in the Micoteca of Universidade do Minho (MUM, Braga, Portugal). Five *Aspergillus*: *A. ustus* PSS, *A. niger* ESH, *A. niger* PSH, *A. niger* AA20, *A. niger* GH1; four Penicillium: *P. pinophilum* EH2, *P. pinophilum* EH3, *P. purpurogem* GH2, *P.* ESS; and one *Mucor sp.* were used.

The screening methodology was carried out preparing agar plates (60x15 mm petri dish) with fucoidan-peptone, fucoidan-urea and fucoidan-sodium nitrate medium. The concentration used were: carbon source 5 $gL^{-1}$, nitrogen source 2 $gL^{-1}$ and agarose 10 $gL^{-1}$ in 100 mM acetate buffer (pH 4.5). Each strain was tested by the tree different treatments; inoculation was realized in the center of the plate and incubation was at 30ºC for ten days (experiments were made by duplicate). The presence of growth were measured each 24 h.

Submerged fermentation was realized in 250 mL Erlenmeyer flask using a minimal medium of fucoidan and urea, 10 and 5 $gL^{-1}$, respectively. Culture medium was sterilized by microfiltration with membranes filter of 0.2 µm. Inoculum was prepared by transferring fungal spores to potato dextrose agar medium, incubated at 30 °C for 5 days. Spores were scraped into 0.01% Tween 80 solution and counted in a Neubauer chamber. The culture conditions used were: $1x10^6$ spores $mL^{-1}$ of inoculum concentration, 30°C, 140 rpm, initial pH 5.0 and incubation time of 96h.

For biomass determination the mycelium obtained after filtration (membrane filter 0.45 µm, 47 mm) was dried at 150 W for 10 -20 min. Total sugar quantification was carried out using phenol-sulfuric acid method (Dubois et al., 1956). The protein concentration in fungal extracts was determined using Coomasie (Bradford) protein assay kit (Pierce).

Enzymatic activity was measured by the dinitrosalicylic acid technique (Miller, 1959) to estimate the release of reducing sugars using the following reaction: 900 µL of sustrate (fucoidan 10 $gL^{-1}$ in 200 mM acetate buffer, pH 4.5) and 100 µL of enzyme extract incubated for 24 h at 37ºC. One unity (U) of enzyme was defined as the amount of enzyme able to releases 1 µmol of reducing sugars per minute. All data correspond to triplicates of independent experiments.

## 3 Results and discussion

Filamentous fungus have the ability to growth over complex substrates behind minimal conditions as well as their capacity to produce a wide range of commercially interesting metabolites. Due to their exceptional high capacity to express and secrete proteins, filamentous fungi have become indispensable for the production of enzymes.

Growth fungal evaluation over fucoidan was carried out with the purpose of found terrestrial microorganism able to depolimeryzate this complex polysaccharide. From the ten strains assesed, testing three diferent mediums, only *Aspergillus niger* PSH, Penicillum *purpurogenum* GH2 and *Mucor sp*., showed mycelium presence in fucoidan-urea plates (Fig. 1) after 24 h of inoculation. However, any strain growth in fucoidan-peptone and fucoidan-sodium nitrate complex. Mucor sp. and *A. niger* PSH colonies cover completely the agar plate after six days of incubation. On the contrary, peripheral growth zone of *P. purpurogenum* GH2 was only a small part of the agar.

The presence of fungal colonies can evidence that these microorganisms are capable to excrete metabolites able to hydrolyze the branched structure of fucoidan, consisting of 1,2-linked fucose residues with sulfate at C-4.

*Aspergillus niger* PSH | *Mucor sp* | *Penicillum purpurogem* GH2

Figure 1. Microorganism with growth presence over fucoidan-urea dishes

Submerged fermentation experiments were realized comparing the strains that showed growing capacity over fucoidan. Urea was selected as the nitrogenum source applied in the culture medium. The morphological form observed during the submerged culture where dispersed mycelial filaments. The particular form exhibited is determined not only by the genetic material of the fungal species but also by the nature of the inoculum as well as the chemical and physical culturing conditions (Papagianni, 2004).
Higher biomass production was 0.84 $gL^{-1}$ with *Mucor sp.* and 0.49 $gL^{-1}$ with *A.* PSH, after 72h (Figure 2a). As was observed in agar plates experiments *P*. GH2 biomass only reach values closed to 0.2 $gL^{-1}$. Substrate consumption was approximately 3.5 $gL^{-1}$ of total sugars.

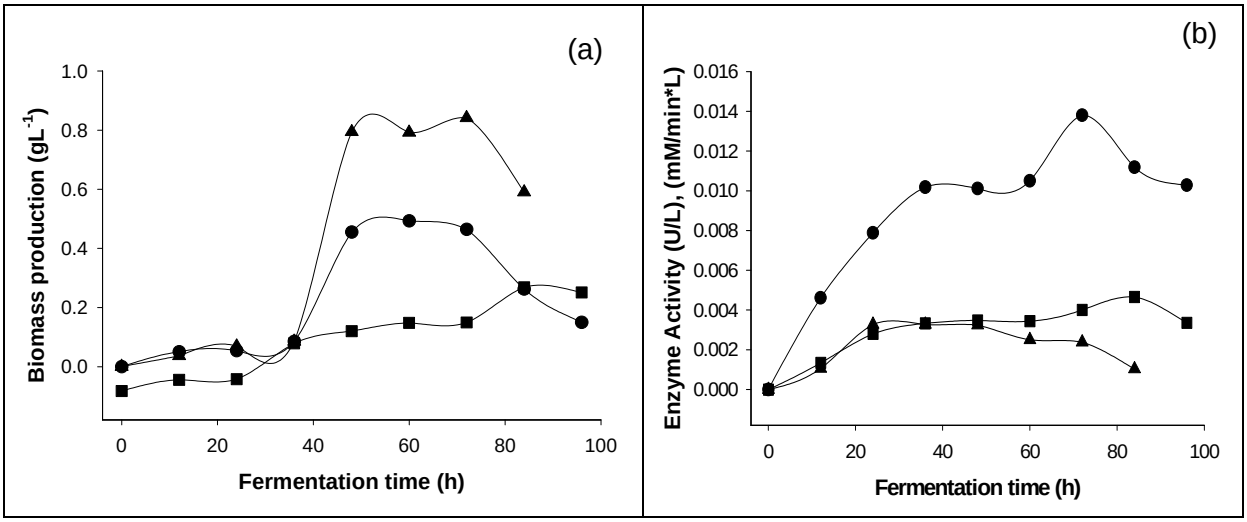


Figure 2. Submerged fermentation with fucoidan-urea medium of *Aspergillus niger* PSH (–●–), *Mucor sp*., (–▲–) and *Penicillium purpurogenum* GH2 ( –■–): a) Biomass production; b) Enzyme activity.

Fucoidan activity (Fig. 2b) obtained by fungal strains was highest with *A.* PSH with values oscillating from 0.0102 to of 0.0138 $UL^{-1}$ after 36h of fermentation. Mucor sp. and *P.* GH2 activities were 0.005 and 0.004 $UL^{-1}$. The enzymatic activity may be underestimated because active proteases were founded at the first 36 h of fermentation, principally in experiments with *P.* GH2.
Despite of enzyme activity were lower, there are any reports of fungal fucoidanases by induction procedure. Morever, fucoidan hydrolases reported by Alexeva et al*.,* (2002), Burtseva et al., (2000) and Bakunina et al., (2000) with marine bacteria and invertebrates exhibited weak catalysis.

The present study suggest that fungal strains from the genus *Aspergillus, Penicillium* and *Mucor* have the capacity for synthesize fucoidan hydrolytic enzymes, being potential microbiology tools for used in fermentation process, looking for the optimization in order to obtain higher titles under different processes.

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