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Identification and Evaluation of Mexican Fungal Strains with Rhamnogalacturonan-Degrading Activity

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Pectinases have been widely used in food and pharmaceutical industry, and their functionality depends on the polysaccharide structural elucidation. Rhamnogalacturonases (RGase) are the enzymes that degrade the hairy pectin region. Mexican filamentous fungus strains were analyzed as possible hydrolyze rhamnogalacturonan enzymes producer. A plaque technique with rhamnogalacturonan dye labeled with remazol brilliant blue was used for RGase activity detection. Radial growth velocities using RG, pectin and glucose, as sole source, were quantified. Enzymes extracts of three strains hydrolyze the dye RG plaque, showing extracellular enzymatic activity. *Aspergillus* FP390 presented the higher adaptation capacity in RG -soy degrading with a duplication time of 0.0683 h^{-1}.

Key Words: Rhamnogalacturonases, Fungal strains, Screening, Hydrolysis.

Rhamnogalacturonases are enzymes which cleaves the main chain of pectins in the rhamnogalacturonan areas, hydrolyzing the α-D-GalA-(1→ 2)- α-L-Rha molecule, RG-hydrolase, or byβ-elimination of a-L-Rha (1→4)-α-D-.GalA -(1→2) molecule, RG-lyase[1].Due to the complexity of pectin hairy regions, where a rhamnogalacturonan backbone is composed to a large extent of alternating 2-linked α-L-rhamnose and 4-linked α-D-galacturonic acid residues, carries side chains (on the O4 position of rhamnose residues) composed essentially of galactose and arabinose residues[2,3], the functionality of this polysaccharide and its oligomers have not been completely understood and exploited for being applied in food or pharmaceutical area.

Filamentous fungi have been widely recognized for being used as produced and isolated source of pectolytic enzymes active toward smooth region of pectin[4]. However, these enzymes have been shown not to be active toward hairy region of pectin. *Aspergillus aculeatus*[5,6] and *Trametes sanguinea*[7] have been founded as producers of enzymes that are able to split the RG backbone of 'hairy regions' commonly isolated from cell walls of different fruit and vegetable sources[8], but also have founded that *Beauveria bassiana* is able to growth over RG and pectin. In contrast, there are

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not reports of use of "black *Aspergillus*" that are highly study in polygalacturonase, pentinlyase and pectinesterase enzymes owing to its GRASS status, high physiological advantages, good fermentation properties that allowed being easy scaling up[9].

Mexico has a high territorial extension with huge microorganism biodiversity able to develop specific characteristics for different ecosystems, with wide levels of adaptation to extrinsic growing factors. For that reason, the objective of this work was to identify Mexican fungal strains from the *Aspergillus* and *Penicillium* genus capable to synthesize pectin hairy region degrading enzymes.

MATERIAL AND METHODS

Microorganism and culture conditions

Four *Aspergillus* and one *Penicillum* strains from the Universidad Nacional Autónoma de México and Universidad Autónoma de Coahuila, respectively, with previous studies of high pectolytic enzymes production were used (Table 1). Strains viability was maintained over agar slant with glycerol at 4 °C (stock culture). For inoculum preparation, culture was grown in potato dextrose agar (PDA) on flask at 30°C for 5 days. Spores suspension concentration was 1×10^8 conidios mL^{-1}.

Table 1. Microorganism with pectinase activity
(SMF submerged fermentation, SSF solid state fermentation)

Code	Microorganism	Pectinase reports
334	*Aspergillus niger* NRRL-334	Collection
FP10	*Aspergillus sp* FP10	Higher production in SSF
FP390	*Aspergillus sp* FP390	Higher production in SSF
FP370	*Aspergillus sp* FP370	Similar production in SSF and SMF
EH3	*Penicillium purpugerum* EH3	Higher production in SSF

The growth medium employed were: carbon source, 10 gL^{-1}; triptone, 5 gL^{-1}; K_2HPO_4, 1.0 gL^{-1}; KCl, 0.5 gL^{-1}; $MgSO_4\times7H_2O$, 0.5 gL^{-1}; $FeSO_4\times6H_2O$, 0.01 gL^{-1}. The initial pH of the media was adjusted to 4.5. The enzymatic extracts were obtained by liquid fermentation at 30 °C, 170 rpm for 45 hours, concentrated by liofilization and suspended with acetic acid - sodium acetate buffer 100 mM, pH 4.5. The carbon source used was Mexican potato (*Pachyrhizus erosus*) rhamnogalacturonan.

Plate screening detection

The substrate employed was Azo-RG-RAB, which is prepared dyeing remazolbrilliant blue with rhamnogalacturonan (Megazyme), 25% (w/v) in acetic acid - sodium acetate buffer 50 mM, pH 4.5, with agarose 1% (w/v). The reaction was carried out in Petri dishes putting 5-10 µL of fermentation enzymatic extracts at 37°C for 24 hours. At the end, Azo-RG-RAB dishes were washed with distilled water in order to eliminate the pigment liberated for the colorant-polysaccharide degradation. Haloes formation over the plaque surface indicates RGase activity.

Assay of rhamnogalacturonase activity

The assay procedure was followed as proposed by Megazyme. Substrate concentration was Azo-RG-RAB 100 mg in 4.5 mL of distilled water, adding acetic acid - sodium acetate buffer 2M, pH 4.5, until reach 5 mL. Colorimetric test was carried out mixing substrate with enzymatic extract (125 µL and 50 µL, respectively) incubating at 40 °C for 24 h. After the reaction, 500 µL of ethanol were added, mixed by vortex and centrifuged at 3000 rpm for 10 min, with the aim of precipitate high molecular weight particles. Supernatant was read at 590 nm.

Radial growth kinetic

Aspergillus FP390, *Aspergillus* FP370 and *Penicillium* EH3 were evaluated using three carbon sources, rhamnogalacturonan and pectin of Mexican potato as inductors and glucose as growth control. Petri dishes with carbon source, 5 gL^{-1}, and agorase, 1 gL^{-1}, were prepared.

The microorganisms were inoculated in the center of the plaque, measuring the radial growth in the four axes each 12 hours until reach 96 hours. The data were adjusted to an exponential equation $y = e^{\mu t}$ and the value of ìmax was quantifying.

RESULTS AND DISCUSSION

Dye-labeled techniques degradation was used as a simple applicable tool for specific detection of rhamnogalacturonase-producing microorganisms, as a certain method that provides preliminary activity quantification because degradation of a high molecular weight polysaccharide will be detected only if the specific polysaccharide is depolymerised[10].

It can be seen in Fig. 1 the Azo-RG-RAB plate with the presence of halos as degradation index. The commercial enzyme NovoFerm 43 (Megazyme) was used as positive control of RGase activity. After 24 hours of incubation at 37 °C was possible to perceive the stained areas formation that was evidenced washing the plate with water in order to diffuse the oligomers formed around polysaccharide-degrading colonies. Strains Despite all the strains growth over the minimum medium only FP370, FP390 y EH3 colonies showed to be surrounding by a halo, bigger for FP370, being an indicator of major amount of enzyme produced in an extracellular way.

Similar behavior was obtained in Megazyme assay (Fig. 2) where the degradation of Azo-RG-RAB was measured calorimetrically. Absorbance values were around 0.19 – 0.21 nm FP370, FP390 and EH3, in contrast with to 0.55 nm of the over-expressed activity of NovoFerm 43.

Growth fungal evaluation over RG and pectin was carried out with the purpose of establishes the potential of depolymerization enzyme production over specific and complex supports. Microorganisms able to adapt to pectic substrates were the same that showed RGase activity in plate, with a highest colony growth rate in *A.* FP390 of 0.0441 h^{-1} and 0.0463 h^{-1} and a duplication time of 15.71 and 14.97 h, RG and pectin, respectively (Fig. 3).

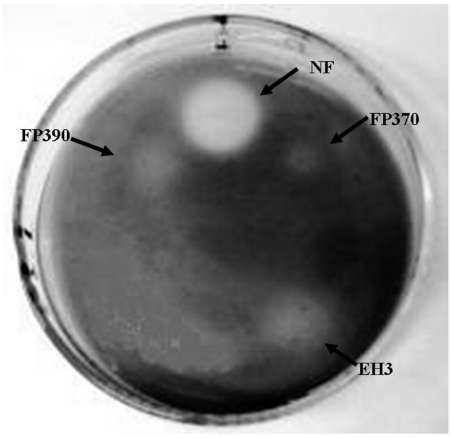

Fig. 1. AZO-RG-RAB plate for fungal rhamnogalacturonase activity detection

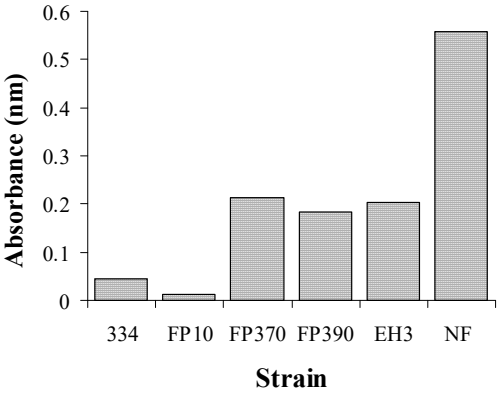

Fig. 2. Absorbance of AZO-RG-RAB solution fungal degradation (Megazyme assay)

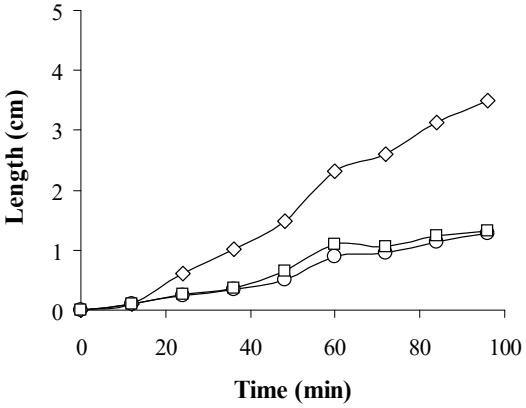

Fig. 3. Radial growth kinetic over rhamnogalacturonan,-♫-FP390, -☒- FP370, -🗹- EH3

CONCLUSIONS

Mexican strains from the genus *Aspergillus* (FP390 and FP370) and *Penicillium* (EH3) were identified with potential for synthesize rhamnogalacturon hydrolytic enzymes, being potential microbiology tools for used in fermentation process.

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