EVALUATION OF CHITINOLYTIC ACTIVITIES OF LECANICILLIUM STRAINS CULTIVATED WITH ADDITION OF HYDROCARBONS AS CARBON SOURCE.

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Abstract.

The aim of this work was the evaluation of degrading capacities and growth of entomopathogenic and mycoparasitic fungi in presence of hydrocarbons. Twenty two strains of *Lecanicillium* and one of *Beauveria bassiana* were cultivated in submerged and superficial media. Pure toluene or n-hexane or their mixtures (in a percentage ratio 17:83) were used, along with yeast extract, glucose or colloidal chitin. According to the results, all the strains of *Lecanicillium* were able to grow using hydrocarbons with or without the addition of other carbon sources (yeast extract). With the presence of hydrocarbons it was observed of the mycelia presenting fine, scarce, in agglomerates with a considerable amount of non germinating spores. The hyphae diameter was significantly smaller with the presence of pure n-hexane. Was measured the n-hexane and toluene consumptions in submerged cultures with or without colloidal chitin of the strains EH-460, 157 and 2149, degradation was determined as 41% to 57%, having bigger toluene degradation. The strain 157 presented the highest consumption (15.4 g toluene/m^3) with a maximum speed of consumption ($7.9x10^{-3}$ g toluene/m^3*h). The activity of endochitinases (Endo) and N-acetil-hexosaminidase (NHase) was determined from the media with or without chitin, therefore these enzymes were also induced by the presence of hydrocarbons. The effect of the toluene with presence of chitin was measured in the strain 2149, these experiments confirmed the expression of NHase and Endo (a maximum activity of 0.27 mU/ml and 11 U/ml respectively) in media without chitin as inducer.

Introduction

The filamentous fungi are characterized by their capacity to colonize hydrophilic and hydrophobic substrates, such as the cuticle of insects. Among the processes involved in the development of entomopathogenic fungi on the cuticle of insects it is found the hyphae formation and other specialized structures that secrete degrading enzymes, such as chitinases, glucanases, proteases, lipases and surfactants molecules, known as hydrophobins[1].

Lecanicillium lecanii (previously known as *Verticillium lecanii*), it is an entomopathogenic fungi recognized as potent producer of chitinases using chitin as inducer[2]. It has been studied in submerged culture (SmF) and solid state fermentation (SSF). SSF has used organic supports as sugarcane bagasse[2] and inorganic supports such as polyurethane[3]. Few reports on the effect of the hydrocarbons on *Verticillium* have been described. It has only been reported the capability of degrading antracene (10 g/l)[4]. N-hexadecane has been used with the purpose of increasing the entomopathogenic capacity of *Beauveria bassiana*, which presented an achievement in its activity against the bean weevil[5]. However there are not studies carried out on the entomopathogenic

activity or related mechanisms, such as chitinolytic activity with the presence of more hydrophobic hydrocarbons such as hexane or toluene and neither their consumption.

Material and Methods

Microorganisms

Twenty-two strains of *Lecanicillium* and one of *Beauveria bassiana* were maintained and cultivated on potato dextrose agar. The strains were employed for the screening based on tolerance, adaptation, degrading capacities of hydrocarbons (n-hexane or toluene) and also their chitinolytic activities.

Screening of fungal strains in submerged culture.

Flasks with mineral medium were used with the following treatments: glucose and yeast extract as control; n-hexane/toluene and yeast extract; n-hexane/toluene. The media were inoculated with $5x10^7$ spores/g of substrate and incubated at 25°C 180 rpm. Biomass by dry weight, hyphae diameters were determined.

Screening of fungal strains in superficial media

The strains were puncture inoculated in petri dishes that mineral agar media with the following treatments: glucose as control; colloidal chitin; colloidal chitin and hydrocarbons; hydrocarbons. Once inoculated the petri dishes were placed in hermetic containers with a tube that contained activated coal and hydrocarbons that was added 1 ml every 2 days. The hydrocarbons that were added were: Toluene, n-hexane or the mixture of both. The containers were incubated at 25°C during 30 days. At the end of the incubation, radial growth, haloes of colloidal chitin hydrolysis hyphae diameters and biomass by dry weight were determined.

Estimation of kinetic parameters of hydrocarbons consumption

L. lecanii EH-460, 157 and 2149 were selected based on their hydrocarbon degrading capacities for the determination of the kinetic parameters of toluene or n-hexane degradation and the effect of hydrocarbon addition on colloidal chitin assimilation.

Mineral media was poured in 125 ml bottles with mininert valves (microcosms), they were inoculated with $5x10^7$ spores/g of substrate and added with the following carbon sources were used: colloidal chitin and toluene (MQT); colloidal chitin and n-hexane (MQH); toluene (MT) and n-hexane (MH). The microcosms were incubated at 25°C and 180 rpm during 80 days, making daily measurements of CO_2 and hydrocarbons (n-hexane or toluene) contents.

Evaluation of chitinolytic activity of *Lecanicillium lecanii* 2149 in mineral medium with added toluene.

L. lecanii 2149 was inoculated in microcosms that contained mineral media with the following carbon sources: colloidal chitin as control; colloidal chitin and toluene; toluene. The media were inoculated with $5x10^7$ spores/g of substrate. The bottles were incubated at 25°C and 180 rpm, measuring biomass (as total protein), pH, N-acetil-hexosaminidase, Endochitinases during 26 days.

Results and Discussion

The twenty three strains inoculated on submerged and superficial media were able to grow using the n-hexane and/or toluene with or without the addition of another source of carbon (i.e. yeast extract) (Figure 1).

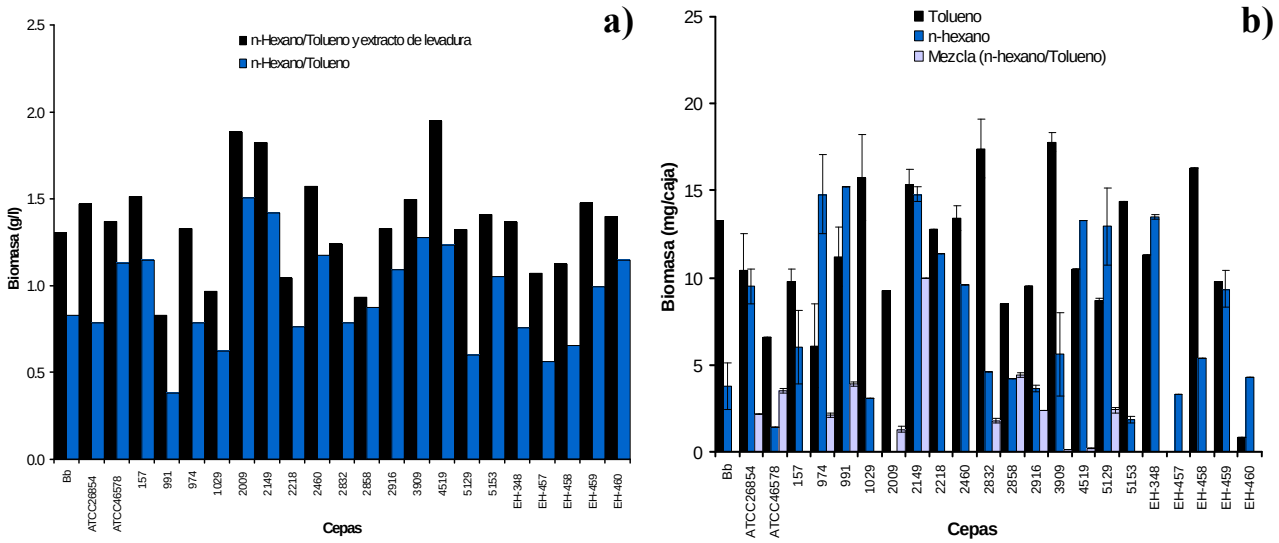

Figure 1. Biomass of strains of *Lecanicillium* in: a) submerged culture and b) superficial media (agar).

Mycelia were observed as fine, scarce, in agglomerates with a considerable amount of non germinated spores. The diameters of the hyphaes were significantly smaller in medium with hydrocarbons (Figure 2).

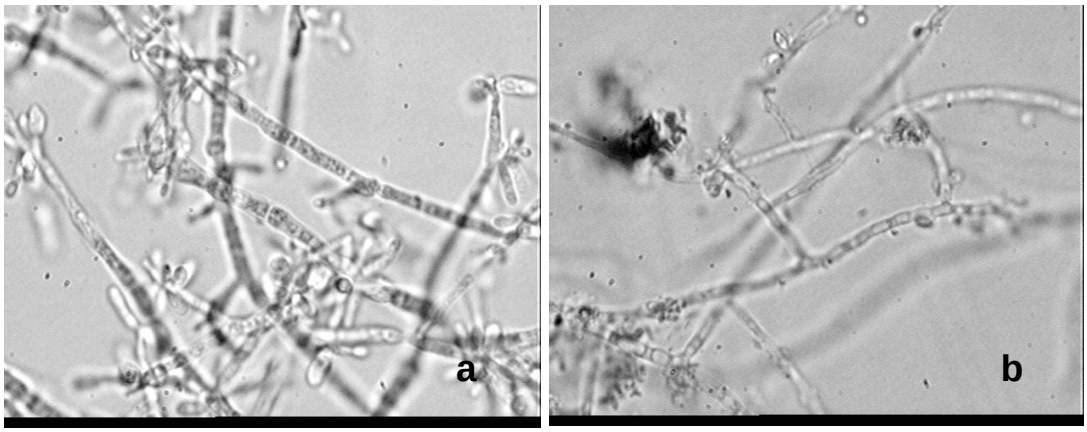

Figure 2. *Lecanicillium* ATCC 46578 in submerged culture to 25°C and 180 rpm, with: a) glucose, b) n-hexane/toluene (100X).

The hyphaes diameters were significantly smaller with the presence of pure n-hexane that with toluene or the mixture of both (Figure 3).

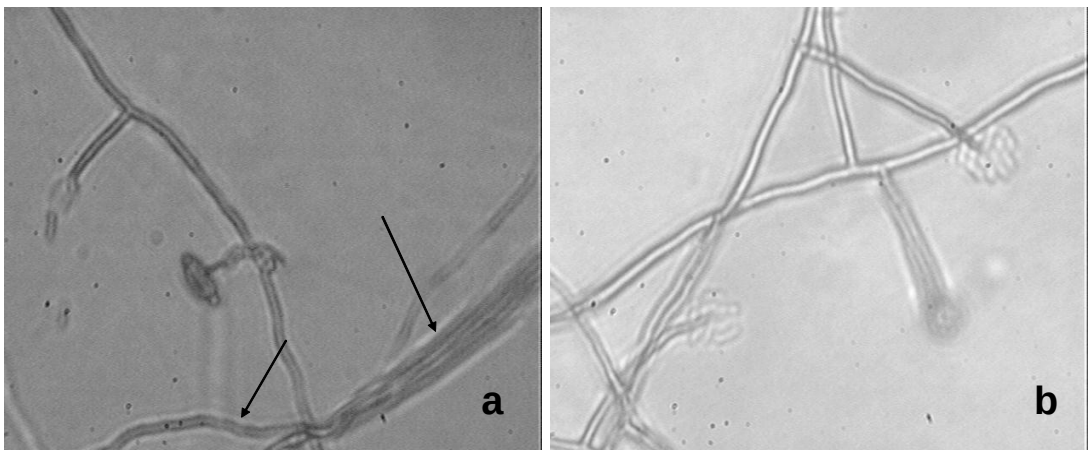

Figure 3. *Lecanicillium* 2149 in superficial medium to 25°C with: a) n-hexane, b) toluene (100X).

The chitinolytic activity was modified by the addition of hydrocarbons, diminishing the radial growth and biomass. The addition of n-hexane reduced considerably the chitinases activities, since only two strains produced of haloes of hydrolysis (Figure 4).

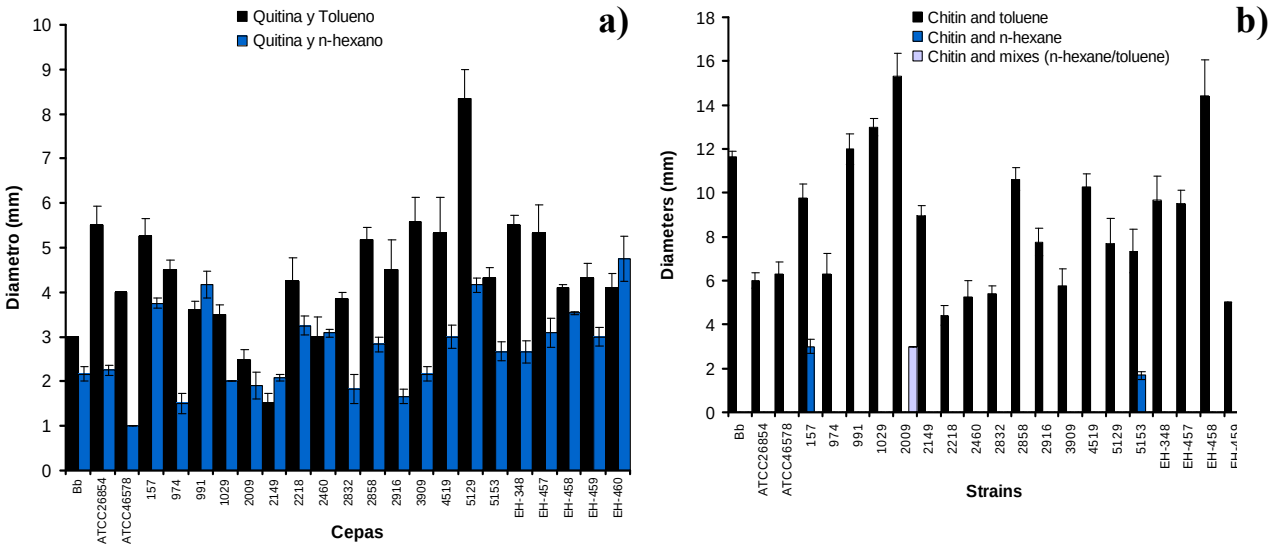

Figure 4. Radial growth (a) and hydrolysis halos (b) of strains of *Lecanicillium* and *B. bassiana* in superficial medium at 25°C.

The n-hexane and toluene consumptions of the strains EH-460, 157 and 214 were measured in submerged cultures with or without colloidal chitin. The degradation was determined as 41% to 57%. The consumption was higher with toluene than n-hexane (Table 1).

Table 1. Kinetic parameters of n-hexane or toluene consumption for *L. lecanii* EH-460, 157 and 2149 in microcosms with mineral medium, with or without colloidal chitin at 25°C and 180 rpm.

Strains	Treatments	Smax (g/m^3)	Vmax ($g/m^3 \cdot h$)
L. lecanii EH-460	n-hexane	25.5	$6.90X10^{-3}$
L. lecanii 157	n-hexane	25.0	$7.90X10^{-3}$
L. lecanii 2149	n-hexane	26.1	$6.10X10^{-3}$
L. lecanii EH-460	n-hexane and colloidal chitin	25.8	$6.40X10^{-3}$
L. lecanii 157	n-hexane and colloidal chitin	26.5	$9.10X10^{-3}$
L. lecanii 2149	n-hexane and colloidal chitin	26.1	$8.60X10^{-3}$
L. lecanii EH-460	Toluene	26.8	$5.40\ X10^{-3}$
L. lecanii 157	Toluene	26.5	$7.30\ X10^{-3}$
L. lecanii 2149	Toluene	26.2	$5.70\ X10^{-3}$
L. lecanii EH-460	Toluene and colloidal chitin	26.3	$7.50\ X10^{-3}$
L. lecanii 157	Toluene and colloidal chitin	26.6	$5.80\ X10^{-3}$
L. lecanii 2149	Toluene and colloidal chitin	27.3	$6.40\ X10^{-3}$

The activity of endochitinases (Endo) and N-acetil-hexosaminidase (NHase) was determined from the media with or without chitin, therefore these enzymes were also induced by the presence of hydrocarbons (Figure 5).

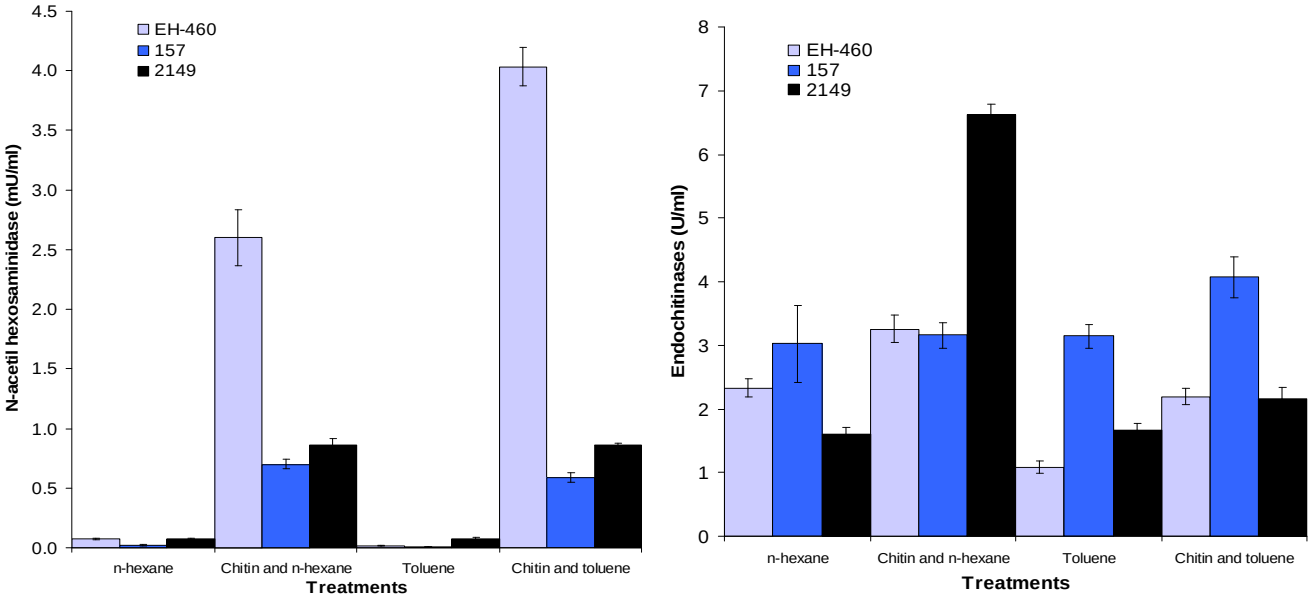

Figure 5. Chitinolytic activities of *L. lecanii* EH-460, 157 and 2149 in mineral medium with or without colloidal chitin and n-hexane or toluene.

The effect of the toluene with added chitin was measured in the strain 2149, detecting a decrease in the growth (Figure 6). The expression of NHase and Endo were confirmed in media without chitin as inducer (Figure 7).

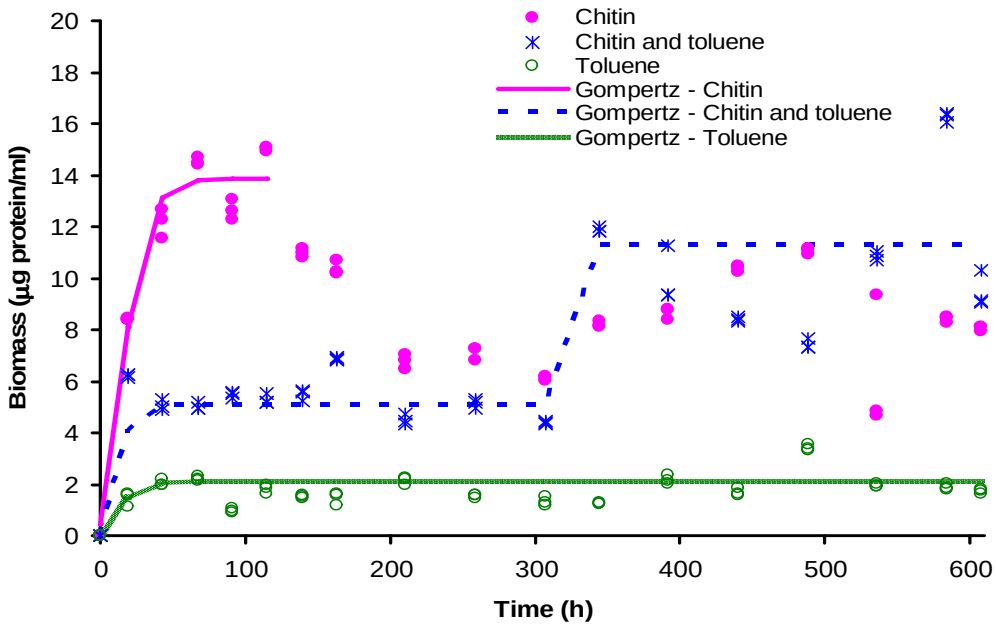

Figure 6. Curves of biomass production of *L lecanii* 2149 expressed as total protein in mineral medium with added colloidal chitin and/or toluene.

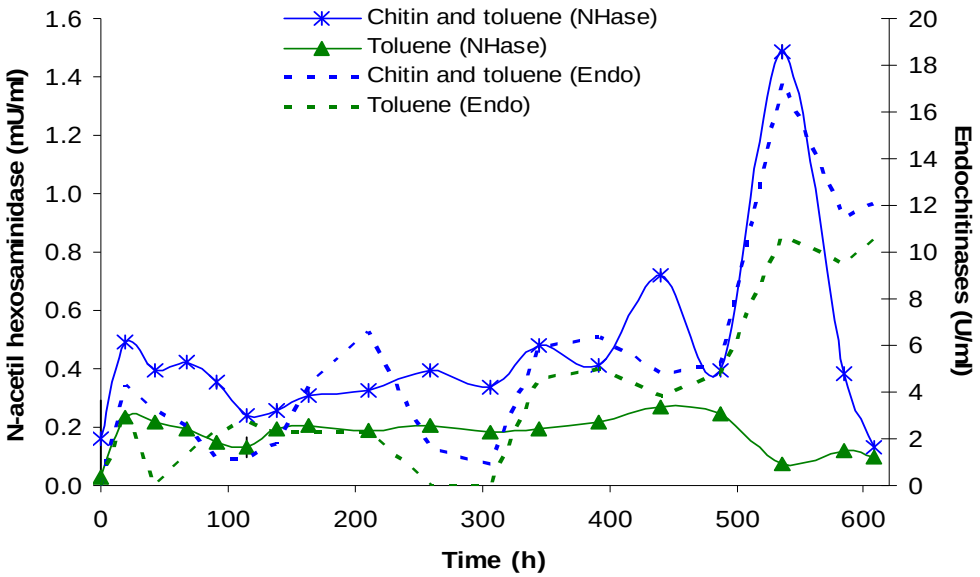

Figure 7. Activities of N-acetil-hexosaminidase and endochitinases of *L. lecanii* 2149 in mineral medium with added colloidal chitin and toluene or toluene as sole carbon sources

Conclusions

The hydrophobicity of the media affected the entomopathogenic fungal growth. The addition of hydrocarbons, especifically toluene, induced mechanisms of adaptation for growth in Lecanicillium, as well as virulence factors such as chitinases.

Acknowledgements

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