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Criteria for the selection of strains of entomopathogenic fungi *Verticillium lecanii* for solid state cultivation

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Abstract

The objective of this study was the selection of strains of *Verticillium lecanii* for solid-state fermentation (SSF) containing cuticle of *Sphenarium purpurascens* as an inducer of proteases and chitinases. The selection criteria were: growth at low water activity (a_w), enzymatic activities (proteases and chitinases) and CO_2 production rate. Three strains of *V. lecanii* were studied ATCC 26854, ATCC 46578 and a wild strain (WS). The strains ATCC 26854 and WS presented the best biomass production at low a_w (0.957). Highest rates of clearing zones of casein and chitin were obtained for strains ATCC 26854 and WS. Best results of CO_2 production in SSF were obtained by using *V. lecanii* ATCC 26854 which showed a maximal value (2.3 mg CO_2 g IDM^{-1} h^{-1}) at 36 h of cultivation. Although clearing zones of casein and chitin were partial criteria for strain selection. It was concluded that growth a low water activities and CO_2 production rate, were more reliable criteria for selecting strains of *V. lecanii* for solid state culture using cuticle of insect as the main C and N source.

Keywords: Solid-state fermentation; Proteases; Chitinases; *Verticillium lecanii*

1. Introduction

Pest biologic control is being considered as an important part of integrated pest management (IPM), which is a more ecological friendly strategy than conventional chemical pest control [1]. For example, using microorganisms such as *Beauveria bassiana, Metarhizium anisopliae, Verticillium lecanii.* The use of transgenic plants containing the *Bacillus thuringensis* toxin gene has raised issues such as the appearance of resistance by pest [2] and a lack of evaluation of its impact on the consumer and the environment [3]. On the other hand the entomopathogenic fungus *Verticillium lecanii* can be used in greenhouses because of to its capacity to infect aphids and whiteflies [4] and in Mexico, the whitefly has become one of most important pests in vegetable crops, resulting in losses for growers.

The infective mechanism developed by the entomopathogenic fungi involves the adhesion of a viscous hydrophilic conidiospore to the external integument of the insect host [5]. The process is presented in two stages: the unspecific adhesion of the spore, mediated by hydrophobic interactions between the walls of the conidium to the epicuticle of resistant insects and hosts [6], allowing the spore to carry out a more specific union. A specific union by lectin association of the spore surface with the insect cuticle has been reported [7]. During the germination of spores *V. lecanii* produces proteases, chitinases and lipases that degrade the cuticle of insects. The penetration is achieved by a combination of enzymatic degradation and mechanical pressure inside, resulting in insect death [8]. Therefore the survival and effectiveness of fungi depend on their capacity to synthesize hydrolytic enzymes [9,10]. Furthermore, infectivity of the fungus is influenced by physical factors such as humidity, temperature and nutrition [11]. High relative humidity is necessary for achieving a good germination and sporulation of *V. lecanii* [12]. St. Leger et al. [13] have studied the infective mechanism in submerged fermentation (SmF) with media containing cuticle or chitin. The model

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consisted in transfering mycelium grown in enriched media to media that contained cuticle or chitin for the induction of the enzymatic systems. Nevertheless, solid-state fermentation (SSF) could be used as a system that mimics the natural behavior of the entomopathogenic fungus. *V. lecanii*, specially regarding the adhesion of conidiospores to the cuticle. Because it has been shown that changes in physiology occur between submerged and solid cultivation [14], this makes it necessary to select strains of entomopathogenic fungi adapted to SSF. Hence, it is convenient to develop an experimental SSF model containing cuticle of insects, for example, *Sphenarium purpurascens*, a common grasshopper, as an inducer of proteases and chitinases.

The aim of the present work was to select strains of *V. lecanii* appropriate for solid-state cultivation, considering: enzymatic activities; the mycelial development at low water activity (a_W); and growth in solid substrate, in terms of CO_2 production. This way SSF and SmF can be compared in terms of proteolytic and chitinolytic activities that may be related to the potential use of entomopathogenic fungus in biologic control.

2. Materials and methods

2.1. Microorganisms

Three strains of *V. lecanii* were used, ATCC 26854, ATCC 46578 and a wild strain (WS) donated by the Dr. A. Trigos from UDLA. Strains were grown in potato dextrose agar (PDA) at 25°C for 7 days and the spores were harvested in a 0.05% Tween 80 solution. They were subcultured in the same medium every 3 months and conserved in glycerol (20% v/v) at −20°C.

2.2. Culture media

Solid state fermentation (SSF) was carried out in glass columns (19 × 5 cm), using sugarcane pith bagasse for support as described by Deschamps et al. [15]. Impregnation medium contained (g l^{-1}): $(NH4)_2SO_4$, 6; $MgSO_4$, 1.2; NaCl, 1; KH_2PO_4, 15; $FeSO_4{\cdot}7H_2O$, 0.1; $ZnSO_4{\cdot}7H_2O$ 0.028; and $MnSO_4{\cdot}H_2O$ 0.032. The cuticle of *Sphenarium purpurascens* (60 g l^{-1}), a common grasshopper, was used as an inducer for proteases and chitinases. The columns contained 35 g of wet matter (WM). The preparation of the cuticle involved removing antennas, paws and head of the insect. The thorax and abdomen were treated with NaOH 0.1 N for 30 min, washed twice with distilled water and once with HCl 0.2 N for 30 min and washed again with distilled water. Resulting material was dried at room temperature, milled, and sifted through a 32 mesh.

The conditions for solid culture were: initial humidity 75%, pH 6 and temperature 25°C. Inoculations were made with 1×10^7 spores (g WM)$^{-1}$·Air flow rate was 0.43 liters of air per kg of WM per minute. Respirometry was evaluated by CO_2 determination in a gas chromatograph (Gowmac Instrument Co, Series 580), as described by Saucedo-Castañeda et al. [16]. Results were expressed as mg CO_2 h^{-1} g^{-1} initial dry mass (IDM).

Submerged liquid culture (SmF) was carried out in 250 ml Erlenmeyer flasks, with 40 ml of medium containing (g l^{-1}): cuticle of *S. purpurascens*, 20; $(NH4)_2SO_4$, 3; $MgSO_4$, 0.6; NaCl, 0.5; KH_2PO_4, 10. Micronutrient concentration was similar to SSF impregnation medium. Cultures were inoculated with 1×10^7 spores ml^{-1}. Initial pH was 6. Incubation was carried out in an orbital shaker (100 rpm) at 25°C during 96 h.

2.3. Growth at different water activities

Strains were cultivated at different water activity (a_W) values in Petri dishes with sucrose 2%, peptone of casein 0.5% and bacteriologic agar 1.5%. To decrease a_W, ethylene glycol (62.07 MW) was used [17] at different concentrations, 5, 10, 15, 20% v/v, resulting in a_W values of 0.997, 0.978, 0.957and 0.933, respectively, as measured by an Aqualab cx-2 analyzer (Labsen Scientific Co). Mycelium was separated from agar, by melting the agar and filtering through a Whatman no. 41 filter paper. Biomass was determined by gravimetry and radii were measured. All experiments were done in triplicate.

2.4. Preparation of enzymatic extracts in SSF

For SSF, the crude enzymatic extract was obtained by mixing the fermented wet material with distilled water 1:1 (w/v). The resulting material was pressed to 1500 psi with a hydraulic press Erkco (Mexican Aeroquip, S.A.). The enzymatic extract was filtered through a Millipore membrane (45 μm) and stored at 4°C for later analysis. For SmF, the medium was centrifuged and the supernatant was filtered and stored as in the previous case.

2.5. Semiquantitative determination of enzymatic activities

Semiquantitative proteolityc and chitinolytic activities were carried out by the formation of clearing zones using casein [18] and chitin [19] as substrates, respectively. A 50 μl aliquot (1×10^4 spores ml^{-1}) was placed in the center of the Petri dish and incubated at 25°C. Radii of clearing zones of enzymatic activity were measured over 9 days of cultivation and the slope was taken as the rate of clearing zones formation. All experiments were done in triplicate.

2.6. Scanning electron microscopy (SEM)

Samples of solid state cultures were fixed, dehydrated and spotter-coated as described by Sarhy-Bagnon et al. [20]. A Carl Zeiss DSM 940A scanning electron microscope was used for examination of the preparations.

2.7. Quantitative determination of enzymatic activities

Proteolytic activity was assayed by the azocoll method [21]. The reaction mixture was prepared as follows, Azocoll (10 mg) was suspended in 1 ml of glycine-NaOH buffer (pH 8.5), 3.9 ml of water and 0.1 ml of enzyme extract were added. Reaction mixture was incubated under agitation (180 rpm) at 37°C for 30 min. An enzyme unit (E.U.) was defined as the amount of enzyme required, resulting in a change of 0.1 unit of absorbance for min^{-1} (g cuticle)$^{-1}$ (ml culture medium)$^{-1}$ at O.D. of 520 nm.

Chitinolityc activity was assayed through a modified method after Coudron et al. [22] using p-nitrophenol N-acetyl-β-D-glucosaminide (Sigma Chemical Co.). The reaction mixture was composed of a 50 μl aliquot of the enzymatic extract, water (150 μl), citrate-phosphate 0.2 M buffer (200 μl), pH 5.6, and 200 μl of substrate (1.0 mg ml^{-1}). Enzyme reaction was carried out at 37°C for 1 h under continuous stirring (180 rpm). Changes in p-nitrophenol concentration were calculated using a calibration curve at 400 nm. An enzymatic unit (E.U.) activity was defined as the quantity of enzyme required to produce a μmol of p-nitrophenol min^{-1} (g cuticle)$^{-1}$ (ml culture medium)$^{-1}$.

For the cellulolytic activity a modified method of Riou et al. [23] was used. 20 μl of the extract was mixed with 1 ml of carboxymethylcellulose (2 mg ml^{-1}) and 1 ml of acetate buffer pH 6. The reaction mixture was incubated at 50°C for 30 min. The liberation of reducing sugars was determined by the 3–5 dinitrosalicilyc, DNS method [24]. Reaction was stopped by addition of 3 ml of the DNS reagent. A unit of cellulolytic activity was defined as the quantity of enzyme required to produce 1 μmol of glucose min^{-1} (g cuticle)$^{-1}$ (ml culture medium)$^{-1}$ at 528 nm. All experiments were done in triplicate.

2.8. pH, protein and elemental analysis

For SSF samples, 2 g of wet material was mixed with 18 ml of distilled water and stirred for 20 min, pH was determined in suspension. For SmF samples pH was determined directly in the media. Protein determination was carried out by the modified method of Lowry [25], using BSA as standard. All experiments were done in triplicate. Elementary analysis of cuticle and sugar cane bagasse was realized by using an elemental analyzer Perkin Elmer Model 2400. Results were expressed as percentage (W:W)

2.9. Statistical analysis

All experimental data were analyzed using ANOVA procedure and different means were compared using the Tukeys test at $P = 0.05$.

Table 1
Effect of a_w on the biomass production [mg cm^{-2}] of *V. lecanii* strains cultivated in Petri dishes after 10 days of cultivation

a_w	Strains		
	ATCC 26854	ATCC 46578	WS
0.997	2.42 ± 0.14	2.51 ± 0.13	2.48 ± 0.1
0.978	2.48 ± 0.07	2.48 ± 0.12	1.94 ± 0.19
0.957	0.97 ± 0.04	0.48 ± 0.1	1.02 ± 0.06
0.93	N.D.	N.D.	N.D.

N.D., no growth detected.

3. Results and discussion

3.1. Growth at low a_W and hydrolysis halos

Table 1 shows biomass production for three strains studied at different a_W levels in Petri dishes. At a_W of 0.997, final biomass production for the three strains of *V. lecanii* was not significantly different, reaching values close to 2.5 mg cm^{-2}, after 10 days of cultivation. However, when a_W was decreased to 0.978, the biomass production for the three strains began to show differences. However, at 0.957 a_W, there were significant differences in final biomass production. Strains WS and ATCC 26854 reached a biomass level close to 1 mg cm^{-2}, while strain ATCC 46578 reached a biomass value of 0.5 mg cm^{-2}. Growth of *V. lecanii* decreased progressively with diminishing a_W values lower than 0.957. None of the strains grew at a a_W of 0.93. These results are similar to those reported by Gillespie and Crawford [26] for *B. bassiana*, *M. anisopliae* and *P. farinosus* when reducing a_W values below 0.970. Water activity was used as a selection criterion in order to simulate a micro environment with low availability of water [27]. It is important to evaluate fungi used in biologic control, growing at low a_W, because their conidia could be ineffective if they are unable to germinate at low a_W [28]. Strains ATCC 26854 and WS presented the best biomass production at low a_W (0.957).

High proteolytic and chitinolytic activities are characteristic of infective strains. These activities were determined by the rate of clearing zone, methodology generally used to select infective strains. Table 2 shows the rate of clearing zone formation due to proteolytic activity in casein-containing media. Strains WS (0.35 cm day^{-1}) and ATCC 26854 (0.32 cm day^{-1}) presented the fastest rates of clearing zone

Table 2
Formation of clearing zones of casein and chitin of different strains of *V. lecanii*, after 9 days of cultivation in Petri dishes

Clearing zones	Strains		
	ATCC 26854	ATCC 46578	WS
Casein	2.875 ± 0.095	2.788 ± 0.11	3.191 ± 0.105
Chitin	1.88 ± 0.13	1.41 ± 0.105	2.07 ± 0.109

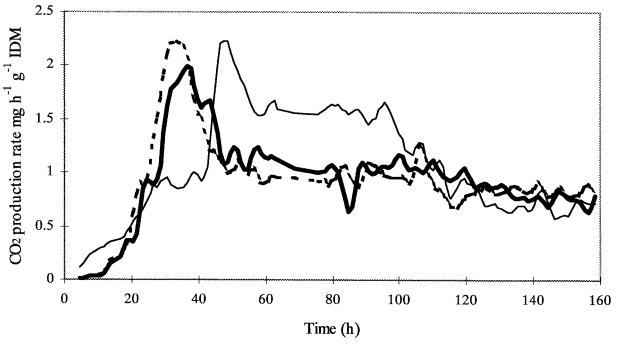

Fig. 1. Evolution of carbon dioxide production rate mg CO_2 h^{-1} (g DMI)$^{-1}$ of different strains of *V. lecanii* cultivated on SSF using cuticle of *S. purpurascens* as inducer. ATCC 26854, (– – – –); ATCC 46578, (————); WS (————).

formation, while the strains ATCC 46578 (0.29 cm day^{-1}) was the slowest. Table 2 shows the rate of clearing zone formation by chitinolytic activity that are comparatively smaller to those of casein. Strains WS and ATCC 26854 present the fastest rates of clearing zones formation with 0.22 and 0.20 cm day^{-1}, respectively. For strain ATCC 46578 the rate was 0.15 cm day^{-1}. Thus WS strain was the most active strain in terms of clearing zones and may be taken as a partial criterion for strain selection [28].

3.2. Estimation of growth through the evolution of CO_2 in SSF

Indirect estimation of growth can be made by on line monitoring of CO_2 production rate which allows the identification of different phases of growth during SSF [29]. Fig. 1 shows CO_2 production profiles of three strains on a medium that contains cuticle of *S. purpurascens.* The ATCC 26854 strain reached a maximum CO_2 production rate of 2.3 mg h^{-1} (g IDM)$^{-1}$, at 30 h of cultivation, followed by the strain ATCC 46578 (17% lower) with a maximum rate of production of 1.9 mg of CO_2 h^{-1} (g IDM)$^{-1}$ at 35 h of cultivation. The WS strain reached a value of 2.3 mg CO_2 h^{-1} (g IDM)$^{-1}$ similar to strain 26854, but at 46 h of cultivation. The CO_2 measured could also be used to estimate losses of dry matter through SSF. After integration of the curves of Fig. 1, the total value of CO_2 produced was similar for three strains: 133.5, 132.6 and 135.7 mg CO_2 (g IDM)$^{-1}$ for ATCC 46578, ATCC 26854 and WS, respectively.

The fact that no cellulolytic activity was found supports use of sugar cane bagasse (C, 45.19; H, 6.03; N, 0.37) as a support for the growth of the fungi. Hence, the only source of carbon for growth was the cuticle (C, 50.84; H, 6.95; N, 10.28) of *S. purpurascens.* In our studies, most of nitrogen was supplied by cuticle (83%) and a lesser fraction by ammonium sulfate (17%). Utilization of cuticle as C and N source involves simultaneous proteases and chitinases activities, these enzymes are characteristic of infective strains [10,25,27].

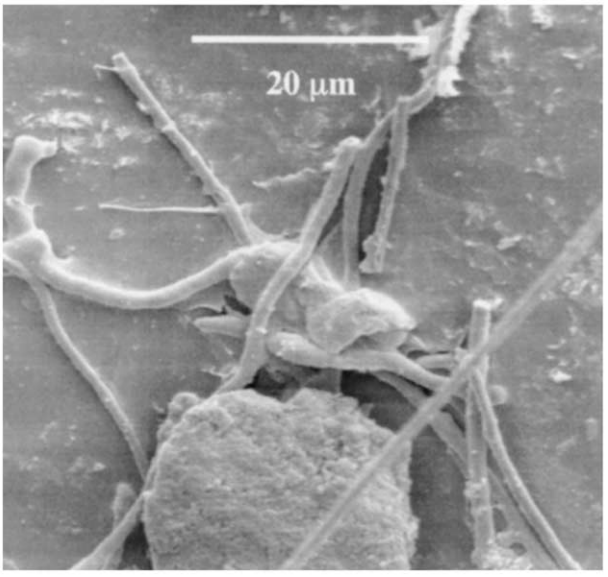

Fig. 2. Appressorium formation of *V. lecanii* ATCC 26854 on cuticle surface of *S. purpurascens* after 24 hours of cultivation in SSF (4 kV, ×750).

The production of CO_2, showed indirectly that the fungi used the cuticle as a carbon source. Nevertheless, CO_2 production may be used as a potential measure of virulence and infectivity, although in vivo assays will be necessary as a direct proof of virulence.

In the present case ATCC 26854 strain presented the highest production rate in the shortest time. However, the WS strain had a similar production profile of CO_2 but reached its maximum rate 16 h later, perhaps because of a delay in the synthesis of cuticle degrading enzymes. ATCC 46578 strain showed lower CO_2 production rates than the other strains. It is worth noticing, that *S. purpurascens* is not a natural host for *V. lecanii,* the spores adhered to it and germinated, possibly because of non-specific hydrophobic interactions [30].

Fig. 2 shows an appresorium formation on cuticle surface, structure observed during infective process [6] allowing to support germ tube on cuticle surface. Fungal penetration can also occur through the mouth and anal opening. Analysis of several samples by SEM indicated that mycelial invasion (Fig. 3) took place on epicuticle surface [31]. These result support the idea that our experimental system could mimic natural behavior in biologic control processes by *V. lecanii.*

3.3. Quantitative enzymatic activities of proteases and chitinases in SSF

V. lecanii grew in media that contained cuticle of *S. purpurascens* in SSF. Selection criteria in the present study were based on the highest proteolytic and chitinolytic activities. The induction of these activities could be related to the results of CO_2 production. In Table 3 the proteolytic and

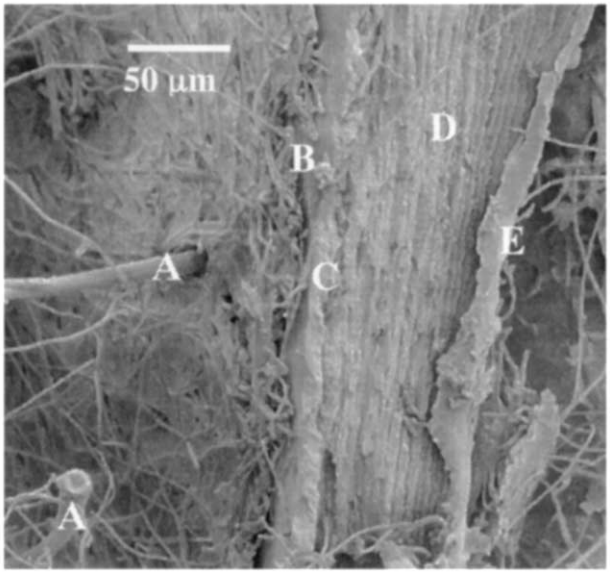

Fig. 3. Growth of *V. lecanii* ATCC 26854 on cuticle surface *S. purpurascens* after 48 hours of cultivation in SSF (4 kV, ×250). A) seta, B) epicuticle, C) procuticle (exocuticle), D) procuticle (endocuticle), E) epidermis.

chitinolytic enzymatic activities of the three strains are presented in SSF at 96 h. Cellulolytic activity was not observed. The ATCC 26854 strain showed the highest values of proteolytic activity, it was close to twice that of the other strains. As for chitinolytic activity, the WS strain presented the highest value being 1.2 and 3.4 times higher than the strains ATCC 26854 and 46578, respectively. The final pH of the culture increased in all cases as a result of proteolytic activity with the liberation of amino groups. At the end of solid cultivation, the values of soluble total protein were similar in all three strains at around 10 mg ml^{-1}. The a_W values at the end of the fermentation were close to 0.99 for all three strains.

Therefore present results indicate different levels of protein breakdown activity when assayed by casein clearing zones or SSF azocoll hydrolysis. Strain WS presented the highest rate of casein clearing zones formation, but the lowest proteolytic activity toward degradation of the cuticle of *S. purpurascens* in SSF. Being casein and cuticle quite different to each other, utilization of complex substrates such as grasshopper cuticle seems to be as a closer indication to the natural system in biologic control by fungus. Thus, strain ATCC 26854 presented the highest proteolytic activity and a chitinolytic activity slightly less than the strain WS. The strain ATCC 26854 presented the best adaptation to SSF, as indicated by the CO_2 production rate.

Table 3
Enzymatic activities obtained in SSF and SmF for the strains of *V. lecanii* after 4 days of incubation

Strains	Enzyme activities (E.U.)			
	Solid state fermentation		Submerged fermentation	
	Proteolytic[a]	Chitinolytic[b]	Proteolytic[a]	Chitinolytic[b]
ATCC 26854	33.9 ± 2.4	37.4 ± 1.7	11.5 ± 0.7	22.2 ± 3.7
ATCC 46578	17.8 ± 2.2	13.5 ± 1.1	11.5 ± 0.2	47.4 ± 2.3
WS	14.9 ± 0.1	46.2 ± 1.1	1.2 ± 0.14	35.3 ± 1.8

[a] (E.U.) The change of 0.1 unit of absorbance for min^{-1} (g cuticle)$^{-1}$ (ml culture medium)$^{-1}$.

[b] (E.U.) µmol of p-nitrophenol min^{-1} (g cuticle)$^{-1}$ (ml culture medium)$^{-1}$.

The differences for the enzymatic activity between the strains ATCC 26854 and WS, would explain the profiles observed in the Fig. 1. Chitin microfibrils are located inside a protein structure, making essential the presence of proteases in order to allow for complete degradation of chitin [28]. Even when WS strain presented high chitinolytic activity, the degradation of the cuticle of *S. purpurascens* was slower.

3.4. Analysis of enzymatic activities in SmF and SSF

The main systems of production of enzymes are SSF and SmF, so we considered it necessary to compare the enzymatic activities of the two different cultivation systems. In this sense, Table 3 shows the enzymatic activities for SmF for three strains of *V. lecanii*. In all the cases the proteolytic activity was lower in SmF. They were 66, 35 and 92% lower for proteolytic activity in the strains ATCC 26854, ATCC 46578 and WS, respectively. For chitin breakdown, the activities were 40 and 23% lower in the strains ATCC 26854 and WS, respectively. ATCC 46578 strain presented the highest level of enzymatic activities among those tested in SmF (Table 3). The adaptability of strains to each culture system, would explain the differences shown in the enzymatic activities for the strains 26854 and 46578. When considering simultaneously the following criteria: a_W, CO_2 production and enzymatic activities, the best strain adapted to express the enzymatic systems of proteases and chitinases in SSF is ATCC 26854. On the other hand, in SmF the strain that shows the best titers in both enzymatic activities is the ATCC 46578. Regarding the WS strain, a better chitinolytic activity and a lower proteolytic activity were observed in SSF (Table 3). It is worth noticing that, both enzyme activities should be present to consider a strain as virulent. The comparison between the two types of fermentation and all the selection criteria allowed the identification of a strain that potentially is best suited for SSF [14]. In this sense, strain ATCC 26854 will be selected for future studies.

4. Conclusions

According to experimental evidence, four different criteria were used for the selection of strains with high proteolytic and chitinolytic activities of the entomopathogenic fungus *V. lecanii*; formation of clearing zones due to enzymatic hydrolysis is only a partial criterion of selection;

growth at low water activities and CO_2 production rate related with the hydrolytic enzyme activities are more reliable criteria. When considering simultaneously all criteria the best strain adapted to express the enzymatic systems of proteases and chitinases in SSF was ATCC 26854. The system of solid fermentation using cuticle of *S. purpurascens* seems to be an interesting model to study the expression and synthesis of degrading enzymes of the cuticle which may be related to the pathogenicity of *V. lecanii*.

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