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Antifungal Activity and the Potential Correlation with Statin-Producing Ability: An Optimized Screening Applied to Filamentous Fungi from Las Yungas Subtropical Rainforest

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Abstract: Research was aimed at investigating statin-related antifungal activity of fungal specimens isolated from Las Yungas Pedemontana Forest. Considering the participation of statins as a potential mechanism for competition in natural environments, their production was particularly analyzed. Following the 'dereplication' concept for identifying natural products, extracts from fungal isolates were screened by yeast-growth inhibition bioassays, TLC and RP-HPLC analysis. Extraction from fungal cultures was performed according to three alternative protocols: glass-beads, Waring blender disruption, or a freeze-thawing method. Waring-blender-obtained fungal extracts led to higher yields of potentially statin-related compounds than those of glass-beads disruption or freeze-thawing. Around a 10% of 201 Las Yungas isolates showed statin-compatible TLC(+) profiles, thus being pre-selected as potential statin-producers. During yeast bioassays, 13 extracts inhibited *S. cerevisiae* ATCC 32051 growth whilst seven had the same effect on *Candida utilis* Pr_{1-2}. Some peaks revealed by RP-HPLC corresponded to known statins like pravastatin, lovastatin and simvastatin. Statin-producing isolates were identified as *Hypocrea* and *Penicillium* genera members. Despite the low statin titres (~3 mg L^{-1}), likely related to non-optimal production conditions, some fungal extracts were able to inhibit HMGCoA-reductase between 1.3 and 18.6%. Antifungal activities of selected isolates could be thus related to statin production, probably as a competition skill in natural ecosystems. This report highlights the hidden biotechnological potential, such as statin production ability, of indigenous filamentous fungi from subtropical rainforests of Las Yungas. Additionally, optimized protocols for statin-production assessment are herein proposed for future screening programs.

Key words: Statins, fungi, Las Yungas, screening, yeast growth inhibition

INTRODUCTION

The incidence of mycotic diseases has seriously increased over last years and a diversity of predisposing factors, such as uncontrolled diabetes, haematologic malignancy,

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transplantations, steroid use, acquired immune deficiency syndrome and the unrestrained use of immunocompromising drugs are currently well recognized (Lorenz and Parks, 1990; Galgóczy *et al*., 2007). Treatments employing amphotericin B or azoles are usually applied as standard therapy, however, the toxicity and frequent side-effects of these drugs are widely known (Lorenz and Parks, 1990; Galgóczy *et al*., 2007). In this context, the importance to respond to the substantial demand for new compounds with antifungal activity has been emphasized (Galgóczy and Vágvölgyi, 2009).

The use of statins as antifungal agents against pathogenic as well as non-pathogenic yeasts was proposed several years ago (Lorenz and Parks, 1990; Chin *et al*., 1997). Furthermore, the therapeutic potential of the combined use of statins in the presence of azoles (Lorenz and Parks, 1990; Nash *et al*., 2002; Song *et al*., 2003; Chamilos *et al*., 2006) and also, with antifungal proteins with defensin-like structure (Galgóczy *et al*., 2007) has been also evidenced. Natural products (NPs), despite competing with other drug discovery products, are still considered promising and often unique candidates in the anticancer and antihypertensive therapeutic areas. Natural statins are among the NPs positioned in the first places of the top-35 worldwide ethical drug sales in the 2000-2002 period (Butler, 2004). Additionally, it has been highlighted that among the top-20 best selling drugs in 1995, six out of them were from fungal origin (Bhilabutra *et al*., 2007).

Statins are compounds either from natural, semi-synthetic or synthetic origin (Huse *et al*., 1998) mainly known for their hypocholesterolemic action by blocking cholesterol biosynthesis at the conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate by HMGCoA-reductase (EC 1.1.1.34) (Manzoni and Rollini, 2002). They are known to exist in the open β-hydroxyacid form (active) as well as in the β-lactone form (inactive) (Samiee *et al*., 2003).

Statins can also block the synthesis of other downstream products in the mevalonate pathway, thus exerting pleiotropic effects (Goldstein and Brown, 1990). The sum of the affected ergosterol biosynthesis (Bard *et al*., 1988; Macreadie *et al*., 2006), the blocked isoprenylation of key signal transduction proteins (Roze and Linz, 1998; Galgóczy *et al*., 2007) and further molecular effects such as the loss of mitochondrial DNA and respiratory deficit (Westermeyer and Macreadie, 2007), can lead to yeast growth inhibition. This antifungal ability may thus be used with therapeutic purposes (Macreadie *et al*., 2006; Galgóczy *et al*., 2007), but additionally, it has been also herein applied as a preliminary statin-production screening tool.

Different filamentous fungi have been reported as statin-producers. Lovastatin production has been described in *Penicillium* sp. (Endo *et al*., 1976a), *Aspergillus terreus* (Alberts *et al*., 1980) and *Monascus ruber* (Endo *et al*., 1976b); mevastatin, in *Penicillium citrinum* (Endo *et al*., 1976a,b) and *Penicillium brevicompactum* (Brown and Smale, 1976), whilst pravastatin could be obtained by mevastatin biotransformation by certain fungi and actinomycetes (Matsuoka *et al*., 1989). Biotechnological challenges in this area may include selecting new producing strains, detecting new statins or improving their production by optimizing cultivation conditions and/or fermentation strategies (Hajjaj *et al*., 2001; Chang *et al*., 2002; Lai *et al*., 2003; Samiee *et al*., 2003; Ahamad *et al*., 2006; Miyake *et al*., 2006; Panda *et al*., 2007; Sayyad *et al*., 2007; Zhuge *et al*., 2008).

In this study, the search for fungal biodiversity with biotechnological potential for statin-like antifungal activity was focused into the phyto-geographical region of Las Yungas, a humid forest located in mountainous areas linked to the Andes and reaching the northwest of Argentina. This ecosystem represents a valuable reservoir of biodiversity. However, as a matter of concern, most of its original area has disappeared and the rest has been modified

by urban and agricultural activities (Anonymous, 2003; Brown and Malizia, 2004; Lodeiro, 2008). In this context, microbiota of Las Yungas is also in danger of disappearance and its biotechnological potential still remains unknown. Screening protocols were optimized through this research in order to investigate the antifungal activity from filamentous fungi isolated from Las Yungas Pedemontana Forest (Tucumán, Argentina) and its potential correlation with statin-producing ability.

MATERIALS AND METHODS

Fungal Isolation Procedure

Samples were collected from Las Yungas Pedemontana Forest (Tucumán, Argentina), between 400-700 m a.s.l. (above sea level), in December 2004. Fungal specimens directly detected by naked eye (mushrooms, cap and shelf fungi) as well as mycelial mats growing on different surfaces were aseptically transferred to sterile flasks. Samples from wood litter, soil, water collections and streams were identically transferred to the laboratory and kept at room temperature until processing.

Small pieces of fungi were immediately cut out, inoculated onto solid medium MYSA (Skaar and Stenwig, 1996) and incubated at 20°C until evident fungal growth. Soil samples or decomposing organic matter were suspended in 45 mL of 0.9% (w/v) saline and kept at 20°C overnight. After gentle shaking, suspensions were serially diluted up to 10^{-4} with 0.9% saline. Liquid samples (water collections and stream water) were similarly diluted. Aliquots of appropriate dilutions were spread onto MYSA surface and incubated at 20°C in order to obtain independent colonies. Periodical subcultures were performed on MYSA plates at 20°C until pure isolates were obtained. Representatives of each different colony morphotype were arbitrarily named and maintained by subculturing (every 4 weeks) on malt Czapek agar plates for 4-7 days at 20°C and stored at 4°C. Fungal strains are all deposited at the PROIMI-MIRCEN fungal culture collection.

Cultivation Conditions and Preparation of Fungal Extracts

Fungal strains were cultured for 14 days at 20°C on lactose-yeast extract agar medium (Vilches Ferrón *et al*., 2005). Organic extracts from 14-day-old fungal cultures were prepared according to three alternative protocols: (1) Glass-beads disruption: mycelium-covered agar plugs were treated with an equal volume of HPLC-grade ethyl acetate plus 2-mm-diam glass beads in screw-capped glass test tubes. Extraction was carried out at 50°C during 20 min, (2) Freeze-thawing: mycelium-covered agar pieces were frozen overnight and, after thawing, supernatant was recovered by centrifugation (7,000× g, 10 min) and mixed with an equal volume of HPLC-grade ethyl acetate. Extraction was performed as in protocol (1) and (3) Waring-blender disruption: the complete solid culture was blended with an equivalent volume of HPLC-grade ethyl acetate in a Waring blender (medium output, 30 sec, 4 cycles). Extraction was performed at room temperature in an orbital shaker at 200 rpm for 2 h.

For the different extraction methods, the organic phase was recovered by centrifugation (11,000× g, 10 min) and concentrated by evaporation under vacuum at room temperature.

Statins Detection

Analysis by TLC was performed in Silica gel $60F_{254}$ aluminum sheets (Merck, Darmstadt, Germany) with benzene:acetone:acetic acid (70:30:3, by vol) as the mobile phase. Active forms of HPLC-grade atorvastatin, lovastatin, mevastatin, pravastatin, rosuvastatin and simvastatin were purchased from Merck and dissolved in HPLC-grade ethyl acetate to

prepare standard solutions. Standard-stock solutions at a final concentration of 8 mg mL^{-1} were stored at -20°C until use. For TLC detection, 8 µL of either 0.4 mg mL^{-1} standard statins or ten-fold concentrated fungal extracts were spotted onto TLC plates. Plates were developed three times with the mobile phase and spots were made visible by staining with iodine vapors.

A preliminary evaluation of the inhibitory effect of statins on the growth of *Candida utilis* Pr_{1-2} (PROIMI-MIRCEN) and *Saccharomyces cerevisiae* ATCC 32051 was performed in liquid cultures. To do this, 10 mL of yeast extract-peptone-dextrose (YEPD) liquid medium were inoculated with 200 µL of 16-h-old yeast suspensions (OD_{550nm} = 0.3). These cultures were incubated at 26°C and 250 rpm for 25 h and periodic samples were withdrawn and analyzed for growth according to both OD_{550nm} and cell counting in a Neubauer chamber. Statin inhibitory effects were evaluated by adding different lovastatin standard concentrations (0.04, 0.2, 0.4 and 0.8 mg mL^{-1}) to culture medium and lovastatin-deprived biotic controls were also grown in parallel.

Plate bioassays were performed by seeding 12 mL of sterile molten yeast nitrogen base (YNB, Difco, Detroit, MI, USA) agar medium (w/o aminoacids and $(NH_4)_2SO_4$, supplemented with 20 g L^{-1} glucose and 0.6 g L^{-1} $(NH_4)_2SO_4$) with 200 µL of yeast suspension, either *Candida utilis* Pr_{1-2} or *Saccharomyces cerevisiae* ATCC 32051 (OD_{550nm} = 0.6). Yeast inoculated medium was poured into a 15-cm-diam glass Petri dish. After solidifying, 6-mm-diam wells were made with the aid of a sterile cork borer and then, 25 µL of each tested sample were poured per well. Increasing concentrations (from 4×10^{-5} up to 0.4 mg mL^{-1}) of all standard statins were evaluated in order to confirm a direct proportionality to yeast growth inhibition on solid medium. For screening purposes, ten-fold concentrated fungal extracts were used. Negative controls only contained the organic solvent (ethyl acetate) and positive controls consisted in 0.4 mg mL^{-1} standard statin solution. Plates were incubated at 26°C for 16 h and afterwards, inhibition haloes were scored.

Those TLC- and yeast-bioassay positive fungal extracts were subsequently analyzed using reversed-phase HPLC (RP-HPLC). Ethyl acetate extracts were first evaporated under vacuum and residues were dissolved in acetonitrile. Standard statins potentially obtainable from natural sources (lovastatin, mevastatin, pravastatin, simvastatin) were dissolved in acetonitrile. RP-HPLC analysis was performed using a Waters e2695 HPLC with a Waters 2998 PDA detector (MA, USA) operating at 237 nm. A 150×4.6 mm Phenomenex Gemini $C_{18,}$ 3-µm particle size column, with an integrated Phenomenex Security Guard C_{18} pre-column, was used. The mobile phase consisting in a mixture of 0.1% (v/v) acetic acid (A) and acetonitrile (B) was eluted at 0.6 mL min^{-1} and temperature was maintained at 30°C. A linear gradient was performed starting with 50% B, rising it up to 100% in 45 min and keeping these conditions until 55 min of run. The UV-Vis peaks spectra were recorded ranging from 200 to 600 nm. When necessary, co-injection of statin standards along with the analyzed fungal extracts was also performed.

Finally, the HMGCoA reductase (HMGCoA-R) inhibition ability of fungal extracts was also evaluated. Waring-blender obtained fungal extracts were tested by using the HMG-CoA Reductase Assay Kit from SIGMA-ALDRICH (CS1090) according to the manufacturer recommendations. Reaction mixture contained 1 µL of ten-fold concentrated extract in a 200 µL final volume. A negative control included the organic solvent (HPLC-grade ethyl acetate). One unit of activity was defined as the enzyme amount required to oxidize 1 µmol of NADPH to $NADP^+$ min^{-1}.

Molecular Fingerprinting

DNA extraction from 4-d-old mycelium aseptically separated from cellophane-covered Czapek agar plates was performed with the Fast DNA kit (Qbiogen, Illkirch, France), according to the manufacturer instructions. Ribosomal DNA regions including the D1/D2 domain of the Large Subunit (LSU) 28S rDNA and internal transcribed spacers (ITS1-5.8S-ITS2) were amplified and sequenced as previously described (López *et al*., 2004; Pajot *et al*., 2007). Sequences were edited with DNAMAN program version 5.2.2 (Lynnon BioSoft, Vandreuil, QB, Canada).

Phylogenetic trees were constructed on the basis of the retrieved validated sequences from GenBank. Sequence comparisons were performed using the basic local alignment search tool (BLAST) program within the GenBank database. The ClustalW computer program was used for alignment of multiple sequences (Thompson *et al*., 1994). Phylogenetic and molecular evolutionary analysis were conducted using MEGA version 4 (Tamura *et al*., 2007).

RESULTS

Extraction Yields from Fungal Cultures Result Improved by Waring-blender-aided Disruption

From 71 samples and three substrates of origin (soil, water and tree-associated samples, e.g., leaves, logs, etc.), collected from eight different sampling sites of Las Yungas Pedemontana forest, 201 fungal specimens (hereafter identified as LY) could be isolated, maintained under laboratory conditions and grown on a reported culture medium for statin production.

Preliminary TLC analysis revealed that around a 10% of these screened fungal isolates showed chromatographic profiles compatible with potential statin-producers (Fig. 1a-c). TLC profiles also evidenced that Waring blender-aided extraction allowed higher recoveries of statin-compatible fungal products than procedures based on glass beads or freeze-thawing disruption. Density of TLC spots in the statin-migration zone was, from high to low recovery level, in the following order: Waring blender>glass beads>freeze-thawing (Fig. 1). Accordingly, Waring-blender obtained fungal extracts were subsequently used for yeast bioassays and RP-HPLC analysis.

Yeast Growth Inhibitory Activity of Fungal Extracts can be used for Bioassay-Guided Statin-Producers Screening

Possible statin-containing samples were further evaluated in their growth inhibitory activity according to yeast bioassays. Preliminary standardization with liquid cultures revealed that yeast growth, both according to OD_{550nm} and microscopic cell counting, was progressively inhibited as lovastatin concentration increased, either for *C. utilis* or *S. cerevisiae* (Fig. 2). Optical density measurements seemed to be slightly more affected than cell counting and, in both yeasts, the highest lovastatin concentrations led to the strongest growth abolishment after around 15 h of cultivation (Fig. 2a-d).

Once confirmed a direct proportionality between statin concentration and yeast growth inhibition, bioassays were also carried out on solid media to check the possibility of standardizing a straightforward screening methodology. All standard statins (atorvastatin, lovastatin, mevastatin, pravastatin, rosuvastatin and simvastatin) were tested at different dilutions on YNB agar. The preferential use of YNB culture medium instead of YEPD for plate bioassays took into account the increased sensitivity previously suggested by Macreadie *et al*. (2006) and Wikhe *et al*. (2007) under low nutrient conditions. Once again,

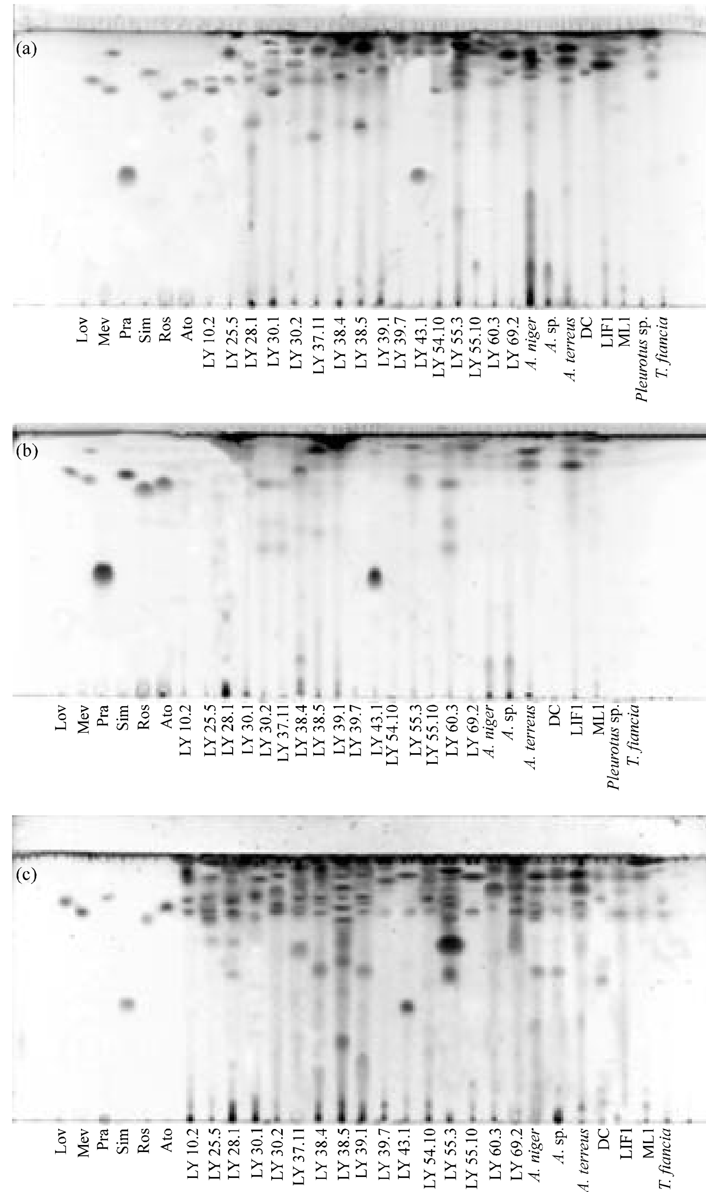

Fig. 1: Detection of statins by TLC. Fungal extracts obtained by (a) glass-beads disruption, (b) freeze-thawing method and (c) Waring-blender disruption, were compared to the standard statins Ato: atorvastatin, Lov: lovastatin, Mev: mevastatin, Pra: pravastatin, Ros: rosuvastatin, Sim: simvastatin. Fungal isolates belonging to the laboratory mycotheque, some of them previously collected from urban and suburban sampling sites, were also comparatively examined. Already identified isolates were indicated as such, whilst in other cases abbreviations were assigned with reference to either the sampling site (e.g., LY denotes Las Yungas isolate; DC, Difunta Correa; LIF, laboratory isolated fungi), or the natural source of isolation (e.g., ML indicates an isolate from melon)

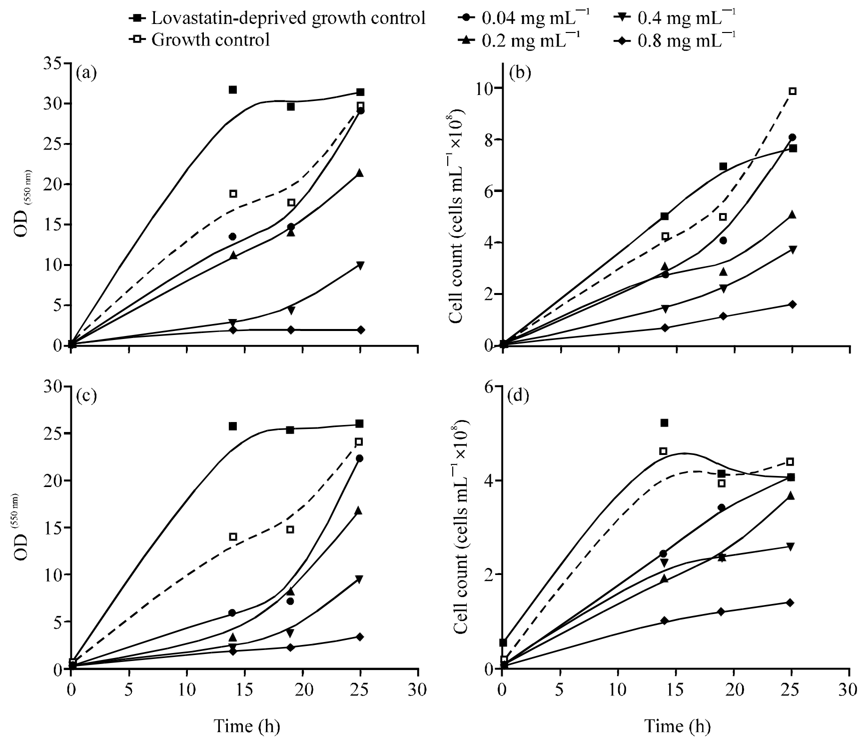

Fig. 2: Inhibitory effects of increasing concentrations of lovastatin on the growth of yeasts *Candida utilis* Pr_{1-2} (a, b) and *Saccharomyces cerevisiae* ATCC 32051 (c, d), as witnessed by Optical Density ($OD_{550\ nm}$) and microscopic cell counting. Standard errors from triplicate experiments were not higher than 10%

the progressive inhibition of yeast growth (both for *C. utilis* and *S. cerevisiae*) as statin concentration increased was corroborated for all tested statins when added to solid culture medium (data not shown).

Semi-quantitative tests indicated that, in *S. cerevisiae*, most of the statins showed a minimal inhibitory concentration (MIC) of 0.04 mg mL^{-1}. On the other hand, *C. utilis* showed to be less sensitive to statins and accordingly, the lowest detectable inhibitory concentration was 0.2 mg mL^{-1} for the majority of tested statins. Only in the case of pravastatin the MIC was 0.2 mg mL^{-1} for both yeasts. Accordingly, bioassays including fungal extracts were systematically performed in comparison to positive controls containing 0.4 mg mL^{-1} standard statin solutions (Fig. 3).

Bioassay-guided screening revealed yeast growth inhibition effects for 18 fungal extracts when confronted to *S. cerevisiae* and for 9 out of the 23 tested extracts in the presence of *C. utilis* (Fig. 3). Thirteen extracts from the former group and 7 extracts from the second one corresponded to LY isolates. It was not unfrequent that the same fungal extract showed a different degree of inhibition against either *C. utilis* or *S. cerevisiae*, for instance in the case of isolates like LY 43.1, LY 55.3, LY 60.3 or LY 69.2 (Fig. 3). Among the mycotheque fungi evaluated for comparison purposes, results from an *A. terreus* isolate, a species generally regarded as a lovastatin-producer (Kumar *et al*., 2000), were especially taken into account (Fig. 3).

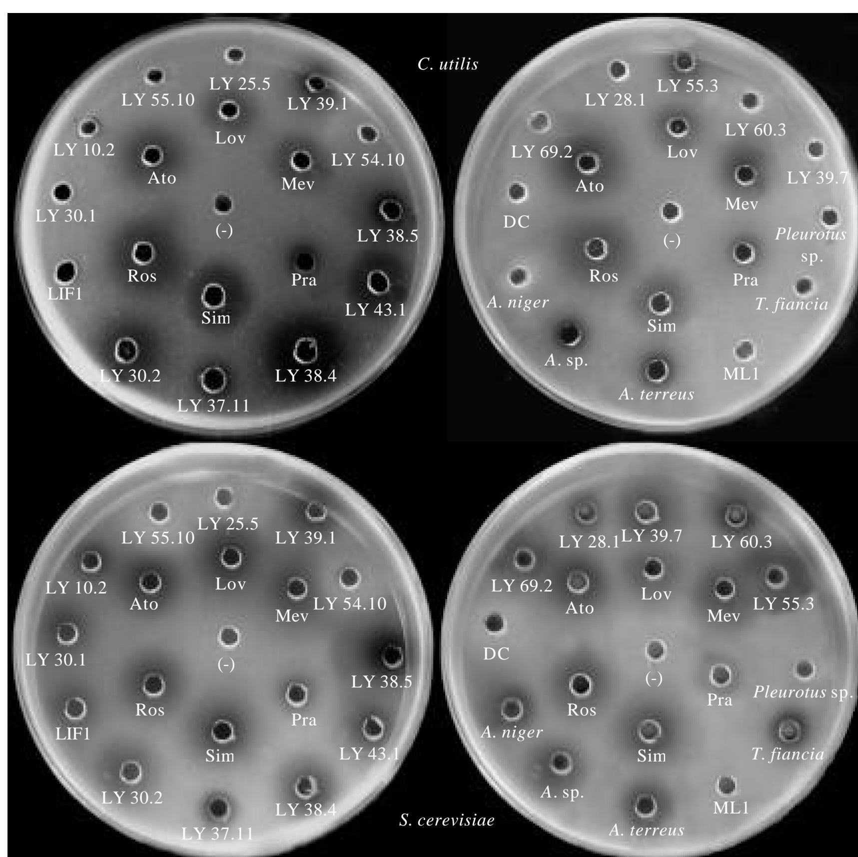

Fig. 3: Bioassay-guided screening in fungal isolates selected at the TLC stage. Inhibition haloes were compared to those produced by standard statins (0.4 mg mL^{-1}). Statin nomenclature is as in Fig. 1, (-) ethyl acetate control, LY: Las Yungas isolates. Non-LY extracts correspond to mycotheque isolates, as described for Fig. 1

RP-HPLC Analysis and HMGCoA-Reductase Inhibition Assay Are in Accordance with Statin Production

Subsequently, the presence of statins was analyzed by RP-HPLC in the different fungal extracts. Peaks corresponding to pravastatin, lovastatin and simvastatin could be detected for some tested extracts at retention times of 6.76, 19.38 and 24.65 (lovastatin hydroxyacid and lactone forms) and 23.78 min, respectively, under the HPLC conditions used. In Las Yungas fungal extracts, statins were encountered at low concentrations (e.g., 2.7 mg L^{-1} pravastatin for LY 39.1) in comparison to the *A. terreus* isolate and in many cases, at non-quantifiable concentrations (Table 1).

Those extracts with a statin-compatible RP-HPLC profile (LY 30.1, LY 37.11, LY 38.4, LY 39.1 and LY 55.3) (Table 1) were further examined in their inhibitory activity according to the HMGCoA-R assay. Some extracts from Las Yungas fungi, despite the low concentrations of statins detected, reached HMGCoA-R inhibition values from 1.3 (for LY 30.1) to 18.6% (for LY 37.11). Unsurprisingly, the *A. terreus* extract led to a 100% inhibition, a predictable

Table 1: Isolates exhibiting statin-compatible profiles in Waring-blender-extracted fungal cultures, according to RP-HPLC analysis

Isolate	Statins detected*
LY 30.1	Pravastatin
LY 37.11	Pravastatin
LY 38.4	Pravastatin
	Lovastatin
LY 39.1	Pravastatin (2.7 mg L^{-1})
	Lovastatin
LY 55.3	Pravastatin
	Simvastatin
A. terreus	Pravastatin (4.8 mg L^{-1})
	Lovastatin (27.0 mg L^{-1})

*Those statins with no annotated concentration exhibited non-quantifiable titres, as measured by RP-HPLC under the conditions tested. Concentrations correspond to culture supernatants

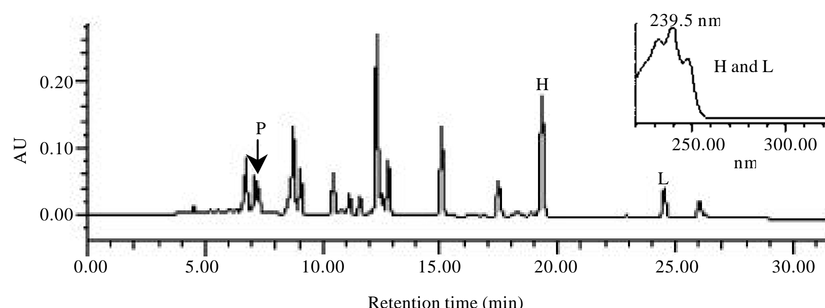

Fig. 4: HPLC chromatogram and UV-Vis absorption spectrum of a lovastatin-containing *A. terreus* solid-culture extract. H: lovastatin β-hydroxyacid form (active), L: lovastatin lactone form (inactive), P: pravastatin β-hydroxyacid form, AU: absorbance units

response according to the HPLC profile (Fig. 4) and the lovastatin and pravastatin titres (Table 1).

The Identity of Promising Isolates Is Disclosed with the Aid of Molecular Taxonomy Tools

Statin producers proceeding from different ecological niches: LY 30.1 (wood log), LY 37.11 (soil), LY 38.4 (mushroom), LY 39.1 (wood log), LY 55.3 (running water) were subsequently identified by means of molecular fingerprinting. Results concerning the phylogenetic affiliations are depicted in Fig. 5. The *A. terreus* isolate was previously identified by conventional taxonomy. Sequences corresponding to the partial 18S ribosomal RNA gene; internal transcribed spacers (ITS1-5.8S-ITS2) and D1/D2 domain of the 28S ribosomal RNA gene were deposited at the GenBank database under the accession numbers: FJ434202 (LY 30.1), FJ434203 (LY 37.11), FJ434204 (LY 38.4), FJ434205 (LY 39.1) and FJ495089 (LY 55.3).

DISCUSSION

The numbers for potential statin-producers in the present work appeared somewhat low when comparing to the literature (Samiee *et al*., 2003). Differences in the prevalence of statin-producing fungi might be related with the ecological procedence of fungal isolates. Likewise, environmental conditions under which fungi have to survive may also exert an influence

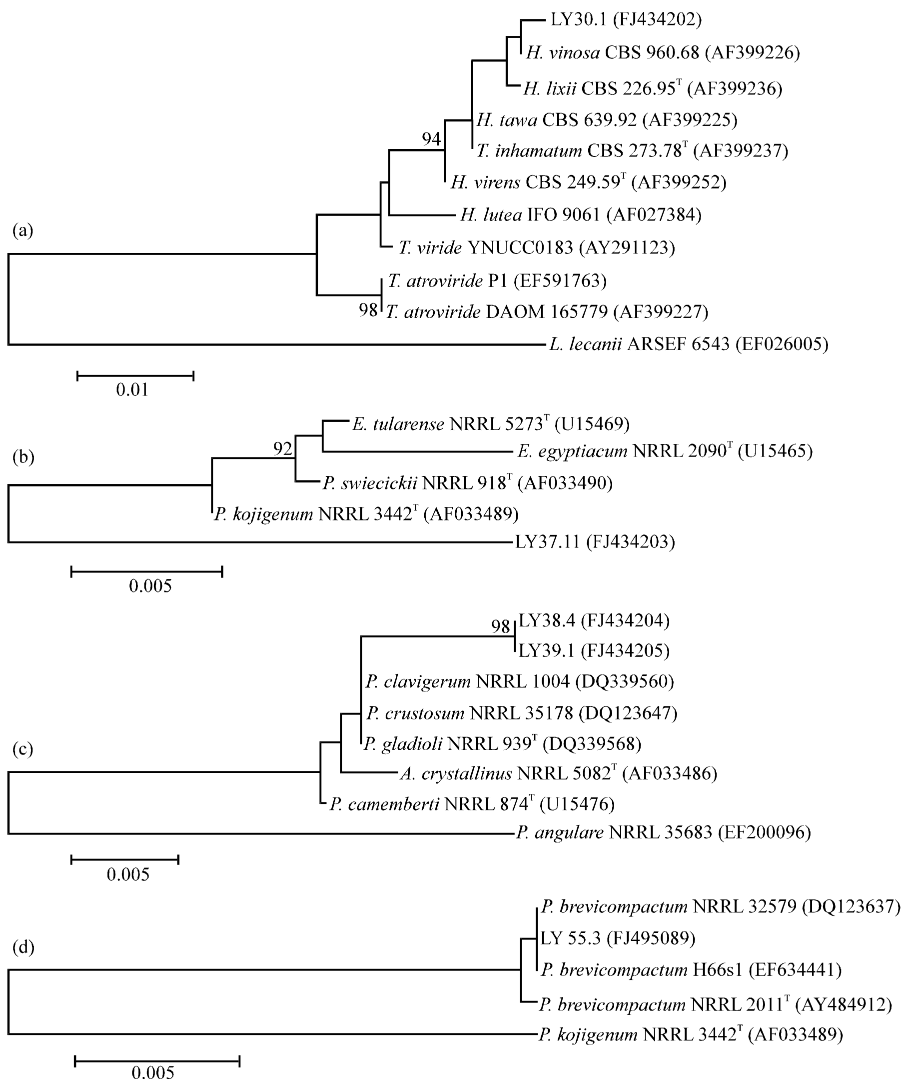

Fig. 5: Phylogenetic trees for the most promising fungal isolates showing the evolutionary history inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (values under 90% were not annotated). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analysis were conducted in MEGA4. (a) LY 30.1, (b) LY 37.11, (c) LY 38.4 and LY 39.1, (d) LY 55.3

(Bhilabutra *et al*., 2007). Statin-producing ability has already been described as a fungal competition tool to overcome adverse conditions such as limited nutrients availability (Gunde-Cimerman and Cimerman, 1995; Macreadie *et al*., 2006; Galgóczy *et al*., 2007).

Another critical step affecting the subsequent statin detection, apart from these natural factors of variance, may be the statin extraction. As already documented, an important amount of statins might be retained in the fungal mycelium and distribution of these compounds in the fungal body may differ according to the species examined (Gunde-Cimerman and Cimerman, 1995; Manzoni *et al*., 1998). In agreement with these reports we found that, among the different extraction procedures tested, the homogenization of complete cultures with Waring blender led to the best results, as witnessed by TLC-analysis.

The progressive inhibition of yeast growth (both for *C. utilis* and *S. cerevisiae*) as statin concentration increased was corroborated for all tested statins when added to solid culture media, thus giving confidence to the bioassay-guided screening. Statin-mediated yeast growth abolishment would be based on the competitive inhibition of HMGCoA-R which, apart from the blocked ergosterol biosynthesis, affects cell cycle progression in other different ways, as above described. Results were in accordance to a previous report on the use of a similar methodology (Vilches Ferrón *et al*., 2005). Conversely, it was in disagreement with research where lovastatin, pravastatin and simvastatin had no antifungal activity in agar-well diffusion assays (Chin *et al*., 1997). As already argumented, the lack of a pro-drug activation step might have been the cause of these previous negative results.

Regarding the improvements on the bioassay methodology, previous protocols (Kumar *et al*., 2000; Vilches Ferrón *et al*., 2005) consisted in the application of paper disks impregnated with fungal lovastatin extracts. In the present study, statins could be directly poured into the agar wells with successful results, making the test simpler and less laborious. A wider spectrum of statins, apart from lovastatin, was also tested. Furthermore, tests could be performed with both *S. cerevisiae* and *Candida utilis* as test strains, giving an idea of the not circumscribed yeast growth inhibitory effects. As a mode of validation, MIC values according to the herein described methodology were in the order of those reported in the literature, which usually ranged between ~0.03 mg mL^{-1} up to 0.2 mg mL^{-1}, depending on the statin or the test strain used (Bard *et al*., 1988; Vilches Ferrón *et al*., 2005; Macreadie *et al*., 2006; Galgóczy *et al*., 2007).

In agreement with the standardization assays with commercial statins, fungal extracts also showed different inhibitory activity if applied to *S. cerevisiae* ATCC or *C. utilis*. Similarly, a varied pattern of yeast growth inhibition was already described even for different *Candida* species when treated with either atorvastatin or simvastatin (Macreadie *et al*., 2006). Present results on both yeast model test strains would be relevant not only to the effects of validating the bioassay-guided screening, but also for a potential therapeutic usage because tested yeasts may behave as opportunistic pathogens (Bougnoux *et al*., 1993; Murphy and Kavanagh, 1999).

The process known as dereplication involves strategies aimed at grouping like-extracts with identical or similar compounds probably responsible for the chemical and/or biological properties observed (Butler, 2004). Under this idea, the search for statin-producing fungi including a bioassay-guided fractionation, constitutes a methodology already recommended for NP drug discovery. This screening tool may be suggested as a preliminary identification of statin-related compounds with antifungal activity.

It has been already emphasized that NP extracts may contain complex mixtures of uncharacterized compounds, some of which may exhibit undesirable properties for the screening purposes, like autofluorescence, UV absorption, or interference with the biological activity evaluated (Butler, 2004). Accordingly, to avoid false positives, results were confirmed by RP-HPLC analysis. This methodology was used as a confirmatory step and not at the beginning, because as already pointed out, a time-consuming isolation strategy only based on peak collection may miss minor active compounds (Butler, 2004).

According to RP-HPLC analysis, some fungal extracts could be associated to the presence of known statins, mainly pravastatin and lovastatin. Additional peaks not yet identified (data not shown), may involve statin-related compounds, either intermediate biosynthetic products (Manzoni *et al*., 1998) or eventually, new statin-structures with minor modifications with respect to statins with closer retention times (Zhuge *et al*., 2008).

Because of statin concentrations found were not very high, the presence of potentiating co-extracted antifungal compounds other than statins was not neglected (Bizukojc and Ledakowicz, 2007; Galgóczy *et al*., 2007). In this sense, the production of peptaibiotics with biofungicide effects has been described in *Trichoderma* species. However, HPLC retention times under similar operative conditions as those herein described were significantly different (Degenkolb *et al*., 2006).

As a mode to check statin-containing extracts, the extinction of NADPH was used to determine the inhibition exerted on the HMGCoA-R activity. To be noted, different statins seem to exhibit a varied Inhibitor Concentration (IC), with values usually ranging from 15-200 nM, depending on the statin nature, the testing model and the form of statin tested (lactone *vs.* free-acid form) (Bard *et al*., 1988; Bischoff and Rodwell, 1996; Gerber *et al*., 2004; Zhuge *et al*., 2008). The IC value may be so relative that for instance, lovastatin showed inhibitory values of 15,000 and 15 nM, depending if the lactone or the free-acid form were tested, respectively (Bischoff and Rodwell, 1996). Significantly high IC values were also described for *Pleurotus* extracts (Gunde-Cimerman *et al*., 1993). It is then expectable that, if produced statins in fungal extracts were not completely converted to the free-acid form (Fig. 4), the inhibition power may have resulted diminished.

Pravastatin production by Las Yungas isolates (Table 1), though still susceptible to optimization, would deserve special attention as it may represent a viable alternative to biotransformation processes (Manzoni *et al*., 1999). Statin distribution may vary depending on the fungal strain or the statin produced. Nevertheless, it was already referred that procedures employing *in toto* cultures instead of the separate mycelium/culture filtrate recovery may lead to low statin yields (Manzoni *et al*., 1998, 1999). Accordingly, experiments aimed at improving statin production by means of optimizing fermentation conditions and statin recovery efficiency are currently in progress.

Preceding results would have revealed the up-to-date unexplored antifungal potential of certain filamentous fungi from Las Yungas rainforest. As recently demonstrated for a *Bionectria* sp. fibrinolytic enzyme-producer (Rovati *et al*., 2010), these indigenous fungal species may represent unappreciated sources of metabolites and/or activities with biotechnological importance. Despite the presumed extensiveness of fungal biodiversity in Las Yungas microflora and the different ecological niches sampled, phylogenetic affiliations of the herein selected microorganisms showed similarity to a discrete number of fungal genera, i.e., *Penicillium* and *Hypocrea,* a *Trichoderma* teleomorph not so far reported for statin production.

Interestingly, the latter genus has been already described as a bioprotective agent against fungal pathogens by means of a very sophisticated and complex mode of action (Degenkolb *et al*., 2006). Some *Trichoderma* species were also previously reported as compactin (mevastatin)-producers, including species such as *T. longibrachiatum*, *T. pseudokoningii*, *T. reesei* and *T. viride* (Samiee *et al*., 2003; Reino *et al*., 2008). However in *T. vinosum* (Jaklitsch *et al*., 2006), the corresponding anamorph to *H. vinosa* (LY 30.1, Fig. 5a), statin production has not been yet reported.

To be particularly highlighted is the possibility to obtain this kind of compounds from a fungal source, since many companies usually prefer microbial extracts as a sustainable drug

supply (Butler, 2004). In addition, an active search for new microbial strains or novel statins has been already emphasized (Zhuge *et al.*, 2008).

CONCLUSIONS

The improved and straightforward methodology proposed for the screening of statin-related antifungal drugs allowed us to disclose the up-to-date hidden biotechnological potential of certain filamentous fungal species residing in Las Yungas rainforest. Results were also accompanied by image documentation on the statin-inhibitory effects. Waring-blender-assisted preparation of fungal extracts, a procedure not so far reported, as well as the optimization of chromatographic techniques were also proved to be useful tools for obtaining accurate and reliable results throughout statin detection and/or quantification. Additionally, critical screening considerations like cost of consumables, time required and people resources could be minimized, making the described protocols valuable tools for a rapid and low-cost search for statin-like or antifungal drugs.

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