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ORIGINAL PAPER

A novel source of fibrinolytic activity: *Bionectria* sp., an unconventional enzyme-producing fungus isolated from Las Yungas rainforest (Tucumán, Argentina)

José I. Rovati · Osvaldo D. Delgado · Lucía I. C. Figueroa · Julia I. Fariña

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Abstract Fibrinolytic enzyme production was evaluated in fungal specimens isolated from the sub-tropical Las Yungas Pedemontana forest (Tucumán, Argentina). Proteolytic and fibrinolytic activities were evaluated in freeze-thaw crude extracts from 230 fungal isolates on 1% w/v skimmed-milk or 0.25% w/v fibrin-agar plates, respectively. Proteolytic activity was positive in 62% of the isolates, whilst only three of them were able to produce extracellular fibrinolytic enzymes on solid nutritive medium. Fibrinolytic-positive extracts were able to degrade fibrin clots in a direct plasminogen-independent way. Selected isolates were identified by sequencing the 26S rDNA D1/D2 domain. Isolates LY 4.1 and LY 4.4 showed a 99.9% similarity with *Bionectria ochroleuca*, while LY 4.2 showed a 99.9% identity with *Cladosporium cladosporioides*. Under submerged culture conditions, LY 4.1 and LY 4.4 were able to excrete fibrinolytic enzymes, reaching a maximum at 120 h of cultivation of 100.2 and 107.9 U/ml in plasmin-equivalent units, respectively. Fibrinolytic enzyme production could be scaled-up to fermenter scale reaching similar values. Fibrin zymography showed that fibrinolytic activity was associated with ~173-, 153- and 80-kDa protein fractions. Extracellular fibrinolytic enzymes from *Bionectria* species may be potentially related to pathogenesis mechanisms, as already demonstrated for serine-proteases from the nematicidal anamorph *Clonostachys rosea*. This work reveals the potential of *Bionectria* strains as an unconventional and unexplored production alternative to already known thrombolytic agents. The value of Las Yungas forests as a reservoir of fungal species with promising biotechnological value could be also highlighted.

Keywords *Bionectria ochroleuca* · Fibrinolytic enzymes · Fungi · Las Yungas rainforest · Screening

J. I. Rovati · O. D. Delgado · L. I. C. Figueroa · J. I. Fariña (✉)
PROIMI-CONICET (Planta Piloto de Procesos Industriales Microbiológicos), Av. Belgrano y Caseros (T4001MVB), Tucumán, Argentina
e-mail: jifarina@yahoo.com; jifarina@proimi.org.ar

L. I. C. Figueroa
Cátedra Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

Introduction

Fibrin is the main protein component of blood clots and is normally formed from fibrinogen by the action of thrombin (EC 3.4.21.5). Blood vessels thrombus due to fibrin accumulation may lead to myocardial infarction or other cardiovascular diseases (Peng et al. 2005). Under physiological conditions, fibrin is lysed by plasmin (EC 3.4.21.7) activated from plasminogen by different plasminogen activators (PAs), keeping blood flow at vascular injury sites (Collen and Lijnen 1991; Kim et al. 2006).

Fibrinolytic enzymes are able to prevent or cure thrombotic diseases by degrading fibrin clots. Typical thrombolytic agents are the tissue-type plasminogen activator (*t*-PA), urokinase-type plasminogen activator (*u*-PA), and bacterial plasminogen activators such as streptokinase (SK). Activators from human origin are generally safe but expensive, whilst others less specific may exhibit undesirable side effects. On this context, searching for new fibrinolytic enzymes from different sources has gained considerable interest worldwide (Hollander 1987; Lu et al. 2007; Ueda et al. 2007).

Various fibrinolytic enzymes have been successively discovered from microbial sources, including bacteria,

actinomyces and fungi (Peng et al. 2005). A variety of enzymes have been also discovered in oriental fermented food products (Mine et al. 2005). Other fibrinolytic principles have been purified from snake venoms (Bello et al. 2006; Maruñak et al. 2004), earthworms (Nakajima et al. 1993; Park et al. 1998) and algae (Matsubara et al. 2000), but most of them are still under study. Mushrooms have been also identified as an important source of thrombolytic agents (Lee et al. 2005).

Filamentous fungi such as *Aspergillus ochraceus*, *Cochliobolus lunatus*, *Fusarium oxysporum*, *Fusarium* sp., *Penicillum chrysogenum*, *Pleurotus ostreatus* or *Rhizopus chinensis* have been found to produce proteases with fibrinolytic activity (Peng et al. 2005). Less common fungi such as *Armillaria mellea* (Lee et al. 2005) and *Cordyceps militaris* (Cui et al. 2008; Kim et al. 2006) have been also recognized as potent fibrinolytic enzyme producers.

The ecoregion of "Las Yungas" is a humid forest occurring in mountainous sectors linked to the Andes that reach the northwest of Argentina. It is one of the environments containing the highest biodiversity in Argentina (Grau and Brown 2000). Despite its flora and fauna biodiversity received great attention during past years, microbial diversity has been scarcely explored (Brown and Malizia 2004). Like many other tropical forests, Las Yungas is at risk because of different human activities which affect their native biota, including fungal microbiota. In this context, diversity restoration as well as the discovery of the biotechnological potential of microbiota, with particular emphasis on fungi, have been previously highlighted as important endeavors (Chaverri and Vílchez 2006).

This work was aimed at detecting the potential fibrinolytic enzyme-production ability in filamentous fungi isolated from Las Yungas Pedemontana forest (Tucumán, Argentina), a stratum playing a key ecological role. Fungal fibrinolytic enzymes, mimicking the ability of enzymes from bacterial origin (streptokinase and staphylokinase) may have potential application in the pharmaceutical industry as thrombolytic agents.

Materials and methods

Fungal isolation procedure

Samples were collected from Las Yungas Pedemontana forest (Tucumán, Argentina), between 400 and 700 meters above sea level (m.a.s.l.). Fungal specimens directly detected by the naked eye (mushrooms, cap and shelf fungi) as well as mycelial mats growing on soils, logs, twigs, leaves, roots, stones, etc., were aseptically transferred to sterile flasks. Mixed samples were also taken from wood litter, superficial soils, small water collections and streams, and identically transferred to the laboratory. Samples were kept at room temperature until processing.

Small pieces of sampled fungi (macroscopically visible) were immediately cut out and inoculated onto MYSA plates (Skaar and Stenwig 1996) containing in g/l: sucrose, 30; malt extract, 15; yeast extract, 5; tryptone, 2; oxgall, 2; $NaNO_3$, 0.5; agar, 20; final pH 6.8 ± 0.2, after adjustment with 1 M NaOH. After sterilization and cooling to 45°C, 10 ml of 0.5% w/v chloramphenicol (Sigma) and 10 ml of 0.5% w/v chlortetracycline (Sigma) were added. Plates were incubated at 20°C until fungal growth was evident. Soil samples or decomposing organic matter (~5 g) 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% w/v saline. Liquid samples (water collections and stream water) were similarly diluted. One hundred microliters from each dilution were spread onto MYSA surface and incubated at 20°C. At the end of incubation, the number of colonies was counted (between 10 and 100 colony forming units, c.f.u.) and average colony numbers were expressed as c.f.u./g sample (wet weight) or as c.f.u./ml sample.

Periodical subcultures were performed on MYSA plates at 20°C until pure isolates were obtained. Representatives of each different colony morphotype were considered an isolate and arbitrarily named. Pure cultures were maintained on malt Czapek agar plates (containing in g/l: malt extract, 40; sucrose, 30; $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.5; agar, 20; initial pH, 4.5), incubated at 20°C for 7 days, subsequently stored at 4°C and sub-cultured every 4 weeks. Isolates were all deposited in the PROIMI-MIRCEN fungal culture collection.

Screening on solid medium

Preparation of crude enzyme extracts

Fungal isolates were grown on 90 mm-Petri dishes containing solid nutrient medium (in g/l): glucose, 35; soybean meal, 20; starch, 10; peptone, 5; meat extract, 5; NaCl, 2; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5 and agar, 20; according to Batomunkueva and Egorov (2001). Plates were inoculated at the center with a mycelium-covered agar plug (~5-mm diam) removed from a 7-day-old malt Czapek agar plate and incubated at 20°C until the whole plate was covered with mycelium (~7–10 days).

Crude enzyme extracts were prepared according to González et al. (1996). Petri dishes totally covered with mycelium were scraped off and the agar was cut into little pieces and put into 15-ml Falcon tubes. Tubes were frozen at −20°C for 10 h, then centrifuged at 7,500×*g* and room temperature for 20 min, and the supernatant was finally

recovered. These supernatants were subsequently used for evaluating enzymatic activities.

Assay for proteolytic activity

Plates containing 1% w/v skimmed milk dissolved in 50 mM Tris–HCl buffer (pH 7.8) as substrate plus 1% w/v agar were used. Ten microliters of each crude enzyme extract were poured into 5-mm-diam wells previously made with an sterile cork borer. After 18 h of incubation, enzyme production was evaluated by measuring halo diameters. For screening purposes, proteolytic activity was expressed as the clear zone area (in mm^2).

Assay for fibrinolytic activity

Fibrinolytic activity was determined according to Astrup and Müllertz (1952), with minor modifications. In Petri dishes, 5 ml of 0.5% w/v fibrinogen (from bovine plasma, Sigma) in 50 mM Tris–HCl buffer (pH 7.8) were mixed with 5 ml of 2% w/v agarose plus 0.1 ml of thrombin (100 NIH U/ml, Wiener® Lab., Argentina). Plates were allowed to stand for 30 min at room temperature to form the fibrin clot layer. Half of these plates were heated at 85°C for 30 min to deactivate plasminogen (plasminogen-free fibrin plates) and remaining ones were considered plasminogen-rich fibrin plates. After this, 10 µl of each crude enzyme extract were dropped into 5-mm-diam wells previously made with an sterile cork borer on each kind of plates.

Plates were incubated at 37°C for 18 h, and fibrinolytic activity was subsequently quantified. A clear transparent region is indicative of fibrin degradation and its diameter is proportional to the potency of fibrinolytic activity. After measuring the diameter of clear zones, the units of enzyme activity were determined according to a reference curve made with varying concentrations of plasmin (from human plasma, Calbiochem, Darmstadt, Germany) as standard fibrinolytic enzyme. Average halo-diam values were computed from two measurements in perpendicular directions, and net lysis areas were corrected by subtracting the area of the well.

All experiments were performed in triplicate.

Identification of isolates

Isolate identification was carried out as previously described (Pajot et al. 2007) by 26S rDNA D1/D2 domain sequence analysis. Sequences were edited by using DNAMAN program version 5.2.2 (Lynnon BioSoft, Vandreuil, QB, Canada). Sequence comparisons were performed using the basic local alignment search tool (BLAST) program within the GenBank database. The ClustalW computer program (Thompson et al. 1994) was used for alignment of multiple sequences.

Phylogenetic trees were constructed on the basis of the retrieved sequences from GenBank of closely related species whose names have been validly published in public databases (GenBank). Phylogenetic and molecular evolutionary analyses were carried out using MEGA version 4.0 package (Tamura et al. 2007) by using neighbor-joining analysis (Nei and Kumar 2000). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. Bootstrap values were based on 1,000 replications; values <90% were not recorded. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

The D1/D2 domain sequences were registered in the GenBank Data Library under accession numbers: EU810762.1 for LY 4.1 isolate, EU810763.1 for LY 4.2, and EU810764.1 for LY 4.4.

Fibrinolytic enzyme production under submerged culture conditions

Inocula of selected isolates were prepared from 5-day-old malt Czapek agar plates grown at 25°C. Five mycelium-covered agar plugs (~5-mm diam) were inoculated into 50 ml of fresh malt Czapek liquid medium and conveniently homogenized with the aid of a Potter–Elvehjem homogenizer. Seed cultures were incubated at 250 rev/min and 25°C for 3 days and then, 10 ml were subsequently inoculated into 500-ml Erlenmeyer flasks containing 100 ml of nutritive liquid medium. Incubation was performed at 250 rpm and 25°C for 5 days; initial pH 5.2. Samples were periodically withdrawn, centrifuged for 10 min at 4,000×*g*, and used as crude enzyme extracts for determining fibrinolytic activity.

In the case of cultivation at fermenter scale, inocula were prepared as described for shake flask scale. A 4-l stirred-tank reactor fitted with baffles and six-flat bladed Rushton turbine impellers (LH Series 210, Inceltech, France) with a working volume of 3 l of nutritive culture medium was inoculated at 10% (v/v). The following conditions were maintained throughout the experiment: air flow rate, 0.3 v/v/min; stirrer speed, 400 rpm; temperature, 25°C.

Fibrin zymography

Fibrinolytic enzymes were recovered from fermenter culture broth after centrifugation at 7,500×*g* and 4°C for 20 min. Samples were analysed by non-denaturing polyacrylamide gel electrophoresis (ND-PAGE, 10% w/v). The protein concentration was quantified by the Folin–Lowry

method using BSA as standard. Post-electrophoretic reactivity after ND-PAGE was visualized as previously described, along with standard proteins, in duplicate (Chitte and Dey 2002). One part of the gel was stained with EZBlue™ (Sigma). The other part was incubated with a fibrin plate for 6 h at 37°C after which, the fibrinolytic activity was evidenced as a clear zone.

Results and discussion

From 71 samples and three substrates of origin (soil, water and tree-associated samples, e.g. leaves, logs, bark, etc.), collected from eight different sampling sites of Las Yungas Pedemontana forest, 201 fungal specimens could be isolated, maintained under laboratory conditions and grown on the selected culture medium for fibrinolytic enzyme production. Isolates were identified as "LY" followed by a serial number corresponding to the sampling site and a subnumber corresponding to the isolation order.

Screening for proteolytic and fibrinolytic enzyme production

Proteolytic and fibrinolytic activities were evaluated in crude enzyme extracts from each tested isolate after growth in nutrient medium agar plates (Fig. 1). For the sake of clarity, only those isolates with outstanding proteolytic and fibrinolytic activities are listed in Table 1. Screening on skimmed-milk agar plates showed that about 62% of the isolates were capable of producing proteolytic activity, whilst only three isolates gave positive fibrinolytic activity.

Skimmed milk-agar plates were used taking into account previous results on the higher activity of fibrinolytic enzymes towards casein than on albumin or other tested proteins (Chang et al. 2000). Fibrinolytic-positive extracts formed similar clear haloes on both plasminogen-rich and plasminogen-free fibrin plates (data not shown), indicating that the enzymes were able to degrade fibrin in a direct way, and lacked the ability to convert plasminogen to plasmin (Jeong et al. 2001; Lu et al. 2007). In contrast with already described *t*-PA, *u*-PA and SK (plasminogen-activator type enzymes), direct fibrinolytic and fibrinogenolytic activity would represent an advantage since undesirable side effects such as platelet activation due to plasmin (Kim et al. 2006) and systemic bleeding (Park et al. 1998) could be avoided.

Counts of fungi from Las Yungas that could produce fibrinolysis were lower than expected, a fact that may be possibly related to the absence of fibrin as a stimulating supplementary nitrogen source in culture medium (Chitte and Dey 2002). As described in most of the recent screening programs with fungal collections (Ueda et al. 2007), the present research was directly focused on the extracellular recovered fibrinolytic activity, considering the potential of large-scale production, recovery and downstream processing.

Fibrinolytic activities were also quantified in comparison to a plasmin standard curve (Table 1). Fibrinolytic activities expressed as clear zones for the selected fungal isolates were quite well positioned as compared to those previously reported (Amatayakul 1955). However, the comparison of fibrinolytic production in plasmin-equivalent units with the literature was not always possible and became somewhat troublesome.

Some pioneer works referring to fibrinolytic clear zones did not compare their results against plasmin (Amatayakul 1955; Park et al. 1998). Others, either expressed fibrinolytic activities as the amount of tyrosine released from fibrin clots (Abdel-Fattah and Ismail 1984; Chang et al. 2000), or eventually converted clear zones to urokinase-equivalent units (Ko et al. 2004; Xiao-lan et al. 2005). Not frequently, fibrinolytic activity was estimated with chromogenic substrates (Chitte and Dey 2002). On the other

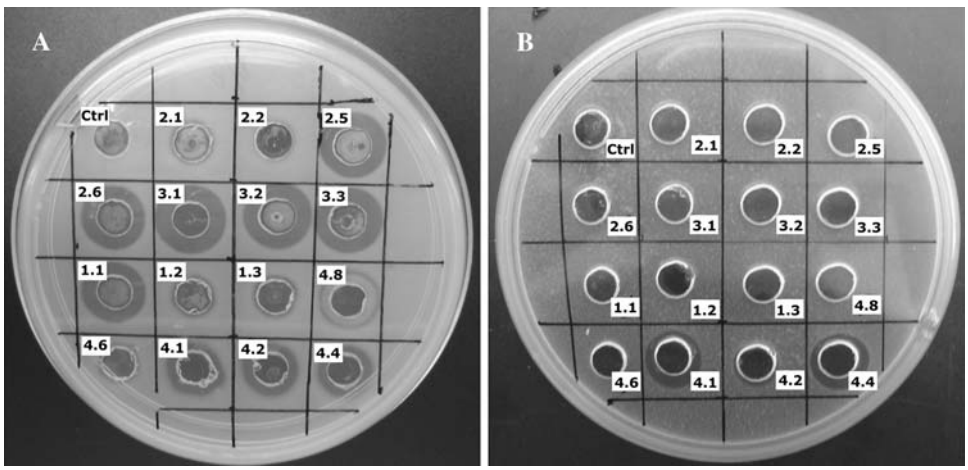

Fig. 1 Screening for enzyme activities in crude extracts from cultures of Las Yungas-isolated fungi. **a** Proteolytic activity on 1% w/v skimmed-milk agar plates. **b** Fibrinolytic activity on 0.25% w/v fibrin agar plates. Highest activities corresponded to the largest clear zones on either skimmed-milk or fibrin agar plates. Numbers correspond to LY isolate identification (see "Results and discussion"). Ctrl: negative control, 10 µl of 50 mM Tris–HCl buffer (pH 7.8)

Table 1 Proteolytic and fibrinolytic activities for the most active fungal isolates according to the screening on solid media

Isolate	Substrate/habitat	Proteolytic activity (mm^2)	Fibrinolytic activity (mm^2)	Plasmin equivalence (U/ml)[a]
LY 2.5	Leaves	94.4	–	–
LY 2.6	Leaves	104.4	–	–
LY 3.1	Decayed woods	94.4	–	–
LY 3.2	Decayed woods	114.8	–	–
LY 3.3	Decayed woods	84.7	–	–
LY 4.1[b]	Decayed woods	84.7	66.7	1.6
LY 4.2[b]	Decayed woods	84.7	21.9	0.3
LY 4.4[b]	Decayed woods	84.7	58.3	1.4
LY 7.1	Decayed woods	50.2	–	–
LY 7.2	Decayed woods	89.5	–	–
LY 33.2	Decayed woods	125.6	–	–
LY 34.2	Decayed woods	62.5	–	–
LY 54.8	Superficial soils	185.4	–	–
LY 54.9	Superficial soils	125.6	–	–
LY 60.3	Roots	136.9	–	–
LY 62.1	Decayed woods	198.6	–	–
LY 62.2	Decayed woods	66.7	–	–

Standard errors from three independent experiments were all below 10%

[a] Plasmin equivalence was calculated according to the plasmin reference curve. For details, see "Materials and methods"

[b] Selected isolates for fibrinolytic enzyme production at the end of the screening program

hand, different works have referred fibrinolytic activities in plasmin-equivalent units (Jeong et al. 2001; Ueda et al. 2007).

Identification of the isolates

In order to identify the selected isolates LY 4.1, LY 4.2 and LY 4.4, sequence analysis of the 26S rDNA D1/D2 domain was carried out. Accordingly, LY 4.1 and LY 4.4 D1/D2 domain showed a 99% similarity with available sequences from *Bionectria* genus, being *Bionectria ochroleuca* the closest relative with 99.9 % identity (Fig. 2a). On the other hand, LY 4.2 D1/D2 domain displayed similarities with *Pezizomycotina* subphylum members, being *Cladosporium cladosporioides* the closest relative with a 99.9% sequence identity (Fig. 2b).

Bionectria sp. isolates have been described from many countries of origin and a diversity of substrates, usually associated with trees, wood or soil. The ecological diversity of this genus is not surprising taking into account its saprotrophic, necrotrophic, biotrophic, or eventually mycoparasitic nature (Hirooka and Kobayashi 2007). The finding of *Bionectria* species in sub-tropical climates seems to have been previously reported (Zhuang et al. 2007).

In particular, *B. ochroleuca* has been collected at high frequency from different localities in Japan (Hirooka and Kobayashi 2007). Although fibrinolytic activity has not been previously reported in this genus, the characterization of an extracellular serine protease from its anamorph *Clonostachys rosea* (Hirooka and Kobayashi 2007; Luo and Zhuang 2007) was described few years ago in a fungus with nematicidal activity (Li et al. 2006).

Fibrinolytic enzymes production in submerged cultures

Following the fibrin-agar plate screening (Fig. 1b), only those isolates with demonstrated fibrinolytic activity were selected for cultivation under submerged culture conditions. Accordingly, LY 4.1, LY 4.2 and LY 4.4 fungal strains were subsequently cultivated in nutritive liquid medium in order to confirm their ability to produce fibrinolytic activity at bench scale. Two of the three isolates were shown to be able to produce extracellular fibrinolytic enzymes under the experimental conditions used (Fig. 3a). For strains LY 4.1 and LY 4.4, fibrinolytic enzyme production started at 48 h of cultivation, reaching maximal values at 120 h of 100.2 and 107.9 U/ml in plasmin-equivalent units, respectively. However, no fibrinolytic activity could be detected throughout this period for LY 4.2 isolate.

Fibrinolytic production values achieved in the present work seemed lower than those previously described by Ueda et al. (2007) for *Fusarium* sp. BLB expressed in plasmin equivalents, and those from Ko et al. (2004) for *B. subtilis* QK02, in urokinase units. However, the values encountered were not distant from those of *Fusarium oxysporum* under solid-state fermentation conditions, expressed in urokinase-equivalent units (Tao et al. 1997). Nevertheless, a factor that will be taken into account for future work, is that glucose present in culture medium may repress fibrinolysin formation (Amatayakul 1955).

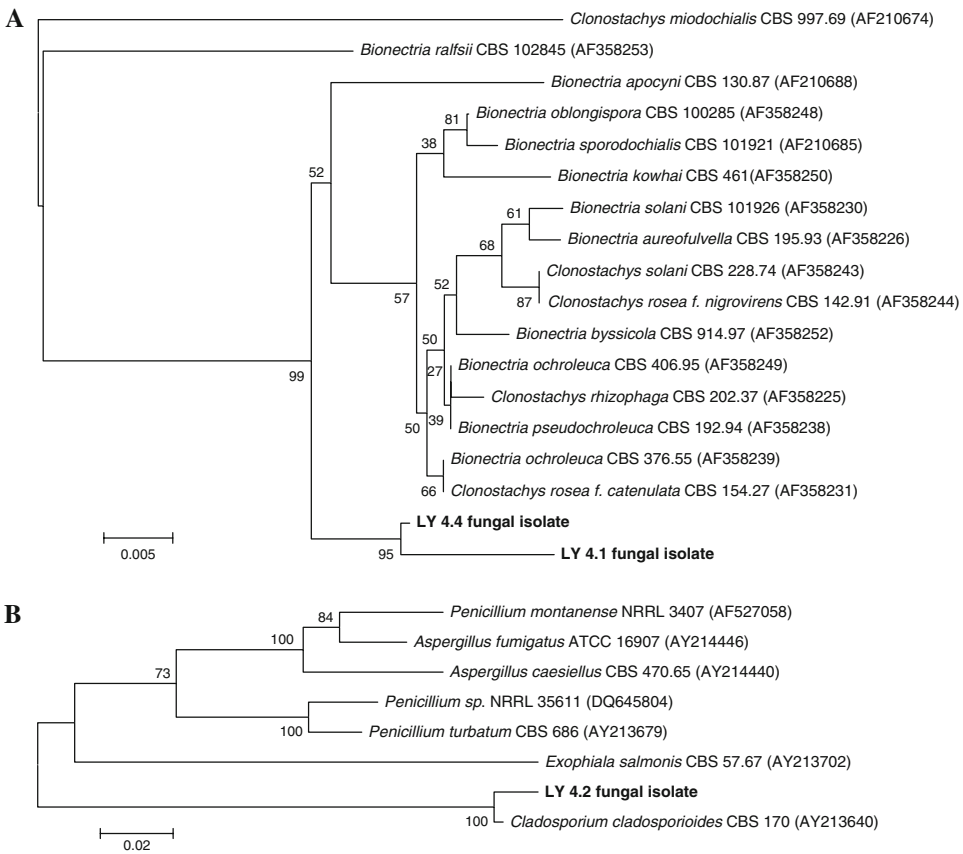

Fig. 2 D1/D2 domain LSU rDNA-based phylogenetic trees showing the evolutionary history inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. **a** Optimal tree for LY 4.1 and LY 4.4 fungal isolates (sum of branch length = 0.07774891). There were a total of 428 positions in the final dataset. **b** Optimal tree for LY 4.2 fungal isolate (sum of branch length = 0.50579558). There were a total of 552 positions in the final dataset

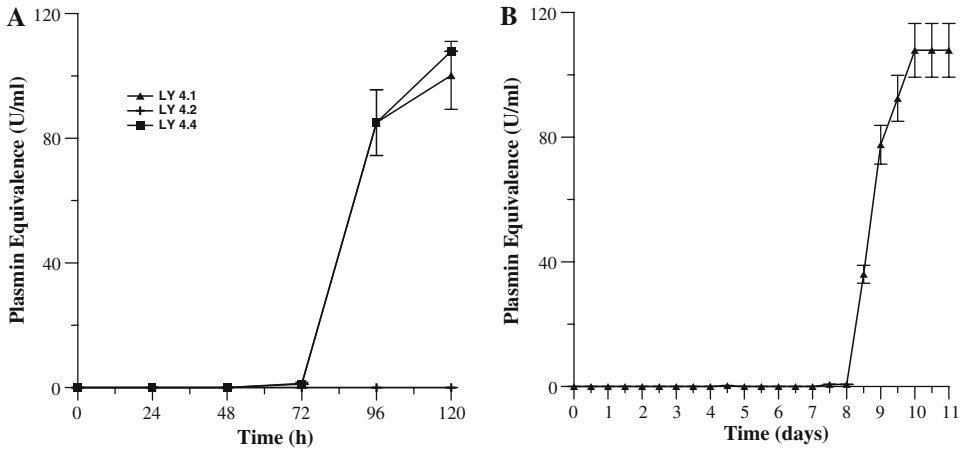

Fig. 3 Fibrinolytic enzymes production with selected isolates LY 4.1, LY 4.2 and LY 4.4 when cultivated in liquid nutrient medium at Erlenmeyer-flask scale (**a**), and for LY 4.1 at fermenter scale (**b**). Enzyme activities were converted to plasmin-equivalent units by using the plasmin calibration curve. For details, see "Materials and methods". Results are average of triplicates from two independent assays

When comparing the behavior of LY 4.2 strain in liquid (Fig. 3a) and solid cultures (Table 1), results were not in agreement. This fact might be attributed to the different cultivation conditions used (Friedrich et al. 1999). On the other hand, LY 4.1 and LY 4.4 produced higher fibrinolytic activity in submerged cultures than those observed on solid media (Fig. 3a, Table 1), revealing that liquid cultures may be not only useful to reproduce the activity observed on solid medium but also to improve it. This property encouraged the subsequent scaling-up.

Results at fermenter scale with LY 4.1 confirmed the ability of this selected isolate for secreting fibrinolytic enzymes when cultured in liquid medium under controlled operative conditions (Fig. 3b). The values achieved, despite similar to those observed at shake-flask scale, implied a longer cultivation time than expected. This fact

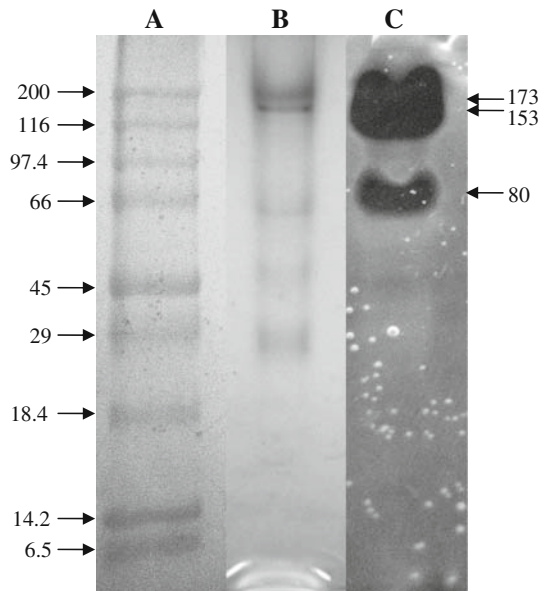

Fig. 4 Non-denaturing polyacrylamide gel electrophoresis (ND-PAGE) and fibrin autography of LY 4.1 crude extract obtained from fermenter culture broth. **a** S8445 SigmaMarker and **b** protein banding profile of LY 4.1 crude extract after staining with EZBlue™ (Sigma). **c** In situ development of crude extract fibrinolytic activity after incubation of the gel on the surface of a fibrin/agarose indicator plate

may be related to the influence of certain operative conditions (e.g. aeration, agitation, etc.) that were arbitrarily set up and may still require optimization.

Fibrin zymography

According to the fibrin autography (Fig. 4), different protein bands identified may be attributed to the fibrinolytic activity of culture supernatant obtained at fermenter scale. When the ND-PAGE was developed against fibrin plates, three highly active clear bands were observed, corresponding to values of ~173-, 153- and 80-kDa. Although these values will be properly confirmed by SDS-PAGE, it could be noted so far that they are larger than the majority of known fibrinolytic enzymes (Peng et al. 2005), but close to the ones reported for *Paenibacillus polymyxa* EJS-3 (Lu et al. 2007).

From all the above results it could be concluded that fibrinolytic activity would be a not very frequent property among wild fungi from Las Yungas Pedemontana forest. This fact might be related to the nature and abundance of the enzymatic substrate (fibrin) towards which this screening program was focused on. Many fungi have the ability to excrete enzymes into their natural environments in a close relationship to the substrates to be attacked, either for nutritional requirements or for surviving purposes (Alves et al. 2002).

A significant number of fibrinolytic enzymes from fungal or bacterial origin have been characterized as serine proteases (Chang et al. 2000; Chitte and Dey 2002; Kim et al. 2006; Ko et al. 2004; Ueda et al. 2007). According to the inhibition profile, this seems to be also the case for the *Bionectria* fibrinolytic enzymes herein described (unpublished data). In accordance, *C. rosea* (Hirooka and Kobayashi 2007; Luo and Zhuang 2007), the anamorph of *B. ochroleuca*, has been referred to as an extracellular serine protease producer (Li et al. 2006).

Nematicidal activity against a variety of earthworms has been assigned to the fungus *C. rosea* (Zhang et al. 2008). The participation of fibrinolytic enzymes in the pathogenesis mechanism of this nematicidal fungus (Li et al. 2006) might be expectable. Different reports have described the purification and characterization of fibrinolytic enzymes from earthworm powders (Nakajima et al. 1993; Park et al. 1998). Therefore, the co-purification of fibrinolytic enzymes from earthworms already colonized by nematicidal fungi would then be not surprising.

Concluding remarks

Plasminogen activators from human origin as well as bacterial fibrinolysins are already available in the market for the therapeutic treatment of thrombotic diseases. Nevertheless, the production of fibrinolytic fungal enzymes, particularly on a large scale, may offer a new, promising and unexploited alternative. *Bionectria* sp., a fungus not reported on this subject until now, may be a potential source of direct (plasminogen-independent) fibrinolytic enzymes for different therapeutic purposes. Accordingly, studies on the optimization of fibrinolysin production and downstream processing with the selected isolate are in progress in an attempt to develop the enzyme for future applications.

From the ecological point of view, the failure to demonstrate the value of tropical forests and their native populations for the benefit of many human activities has debilitated the efforts to conserve biodiversity. Therefore, the discovery of biotechnologically important specimens would not only promote the recolonization of degraded or deforested areas, but also the preservation of potential sources of natural products. Considering that around 95% of fungal species remain still undiscovered, and that many of them reside in tropical regions (Chaverri and Vílchez 2006), screening programs aimed at revealing the hidden biotechnological potential in risk of disappearance would be highly relevant.

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