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Pool of Biological Resources for Potential Applications in Solid State Fermentation Obtained from a Forest Plantation of *Pinus pseudostrobus* Lindl, Mexico

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Abstract

A forest plantation, product of the reforestation of pine trees, represented a pool of biological resources for the implementation of a solid state fermentation process. The trees were identified as *Pinus pseudostrobus* Lindl from which lignocellulosic material in the form of pine needles was collected. Soil fungi, responsible for plant litter decomposition, were cultured at laboratory conditions and tested for their ability to grow on cellulose and hemicellulose as the sole carbon sources. A fungal strain, belonging to the genus *Penicillium*, was selected for growing it on pine needles as the substrate in a solid state culture. After following the culture for six days, the newly isolated strain exhibited a much higher capacity for spore production and holocellulose degradation, compared to a purchased strain of *Penicillium chrysogenum* and two control conditions. This work marks the beginning of future studies focused on commercial applications and represents the first report of a biotechnological process based on pine needles and their degradation by an ascomycetes species belonging to the genus *Penicillium*.

Keywords

Forest Plantation, Solid State Fermentation, Lignocellulosic Material, Pine Needles, Plant Litter Decomposition, Soil Fungi

1. Introduction

Forest ecosystems cover about 30% of the land surface and provide products to society, which include wood, fibers, edible and medicinal plants, as well as environmental services such as watershed protection, carbon sequestration, conservation of biodiversity, scenic beauty, and recreation, among others [1] [2] [3]. For these reasons, it is important to study and understand these systems in order to make a correct and sustainable use of them. Forest dynamics depend on matter transformations and energy flow between components, which in turn, according to Coleman *et al.* [4], must be understood from four perspectives: productivity, consumption, decomposition, and abiotic components.

On the surface of the soil, dead tissues derived from primary production are continuously deposited and accumulated. These residues are part of soil litter, which defined by Spurr and Barnes [5], consist of structures still recognizable as can be leaves, twigs, fruits, and dead animal corpses; its availability is generally mediated by the type and abundance of vegetation. Decomposition of plant litter is performed through interactions within food webs, which represents an essential process for nutrient cycling and regeneration, comprising physical, chemical, and biological mechanisms that transform organic matter into increasingly stable forms [6] [7] [8] [9]. Primary decomposition may be accomplished by abiotic (rainfall, temperature, and humidity), as well as biotic factors (bacteria, fungi, and soil fauna) [10] [11]. In nature both bacteria and fungi are able to break down the main components of plant litter: cellulose, hemicellulose, and lignin, commonly known as lignocellulosic material.

According to Gernandt and Pérez de la Rosa [12], four of the six conifer families (Cupressaceae, Pinaceae, Podocarpaceae, and Taxaceae) are present in Mexico. Coniferous forests represent 15% of the Mexican territory [13], an area that includes the *Sierra* region and the Trans-Mexican Volcanic Belt. National Park *Insurgente Miguel Hidalgo y Costilla*, popularly known as *La Marquesa*, is located in the Trans-Mexican Volcanic Belt, between *Estado de México* and *Distrito Federal*, approximately 50 km West of Mexico City and at an altitude ranging from 2700 to 3100 m. Climate can be classified as temperate with dry winters (Cw according to Köppen), exhibiting annual average temperatures of 12˚C - 14˚C and possibilities of snowfall during January and February. Flora consists mainly of grasses and conifers, the latter covering the soil below them with abundant plant litter.

Due to their high intrinsic capability of decomposition, saprophytic fungi were selected as model for lignocellulosic breakdown, therefore, our research group decided to isolate fungal strains from forest soil. The area of study was located at *La Marquesa*, near the town of *Salazar* (**Figure 1(a)** and **Figure 1(b)**, [14] [15], respectively), with the following geographical coordinates: 19˚18'9"N, 99˚23'56"W. This site consists of a plantation of pine trees derived directly from reforestation (**Figure 1(c)**), and its soil floor is densely covered with dried pine needles which almost account for the total plant litter. Pine litter, as a potential substrate for solid state fermentation (SSF), a process in which microorganisms grow on solid materials in the absence of free liquid [16] [17], was collected from the uppermost organic (O) horizon (Oi). On the other

Figure 1. Location of *La Marquesa* National Park (a) with a more detailed view of the area of study, encircled in red (b), which represents a forest plantation (c). Map images were obtained from Antartis and Google Maps [14] [15].

hand, the complete O horizon (Oi, Oe, and Oa) [18] [19] represented the microbial source. With the combination of these elements (plant litter and fungal strains), the aim of the present work is to characterize the site of study and to establish a SSF process based on the biological resources obtained from it. The findings derived from this research might represent the starting point for a wide variety of future studies. An ecological approach might be followed in order to identify microbial communities, responsible for litter degradation, and the genetic biodiversity associated to them. The enzymatic machinery, result of this diversity, and other metabolic products are interesting targets for their potential and at the same time, sustainable commercialization.

2. Materials and Methods

Methods described below were developed in our laboratory; otherwise, previous reported works are indicated by proper citation.

2.1. Forest Plantation Characterization

Pine trees characterization was performed by collecting cones and foliage. Preservation of leaves was accomplished by spreading them on flat sheets of newspaper for further drying in a plant press. Once dried, specimens were mounted on sheets of stiff white paper and labeled with essential data. Not mounted material such as pine cones was stored separately in cardboard boxes. The identification of the pine trees was accomplished by comparing the collected material with named specimens, photographs, and illustrations deposited previously in the *Herbario Nacional de México* (MEXU) of *Instituto de Biología* (U.N.A.M.).

A circular plot for sampling an area of 1000 m^2 was established in the forest plantation described above. General data such as location, geographical coordinates, altitude,

slope, and vegetation type were registered. For determining plant litter volume and surface area, a planar intersection method was followed, in which strings were oriented to 0˚ (North facing), 120˚, and 240˚. Care was taken in order to avoid pressing the dead biomass on the ground around the ropes. The depth of forest residues was measured with the aid of a steel ruler at distances of 5, 10, 15, 20, and 25 m from the center. In each of the five points, all of the litter contained in a quadrant of 30 × 30 cm was removed and collected in paper bags for drying and transfer to the laboratory, with the aim of calculating the amount of plant material available. In addition, all the trees inside the sample plot were counted.

2.2. Pine Needles Collection, Pretreatment for SSF, and Characterization

Plant litter was collected during February and March, which represent the time of year with less precipitation. This season was chosen in order to avoid a further drying process of the potential substrate for SSF. Pine needles were manually collected in polypropylene sacks of 50 kg capacity (54 × 100 cm) and transported to *Ciudad Universitaria* in Mexico City. Moisture content of pine needles was determined by drying the sample at 60˚C until constant weight, and their length was measured with a ruler, exhibiting values between 15 and 30 cm. Therefore, size reduction was performed first, by cutting the needles with metal scissors and then, by grinding (Cullatti AG mill). Fractionation of particles by size was accomplished using standard wirecloth testing sieves with the following ASTM (American Society for Testing Materials) designation and ISO (International Standards Organization) specification in parenthesis: 18 (1.0 mm) and 35 (500 μm). Lignin and polysaccharides (cellulose and hemicellulose) contents of the major fraction derived from sieving were determined according to the method reported by Van Soest [20], which is based on detergent extraction.

2.3. Fungal Strains Isolation

In order to collect spores, 10 g of decaying plant litter were submerged and vigorously stirred (Thermolyne Cimarec 1 magnetic stirrer model S46415, 15 min at 600 rpm) on a Tween 80 solution (0.01% v/v). The resulting suspension was subjected to serial dilutions, by repeatedly mixing 1 mL of sample to 9 mL of saline solution (NaCl 0.85% w/v) until a 10^{-6}-fold factor. The tubes corresponding to the original sample and to 10^{-2}, 10^{-4}, and 10^{-6} dilution factors, were plated and incubated at 29˚C on Petri dishes which contained standard nutrient medium (SNM), previously reported by Zorn *et al.* [21] for fungi screening. This culture medium has the following composition (per liter): 30.0 g glucose, 4.5 g asparagine, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$, 3.0 g yeast extract, and 1.0 mL of trace elements solution, which in addition contains (per liter) 0.08 g $FeCl_3{\cdot}6H_2O$, 0.09 g $ZnSO_4{\cdot}7H_2O$, 0.03 g $MnSO_4{\cdot}H_2O$, 0.005 g $CuSO_4{\cdot}5H_2O$, and 0.4 g Titriplex III EDTA. For solid media, agar was employed at a concentration of 15 g per liter. Due to its broad spectrum for hindering bacterial growth, chloramphenicol was added to the medium at a concentration of 0.05 g per liter. Distilled water was employed in all formulations, and the pH was adjusted to 6.0 with NaOH 1N. Based on their morphological differences,

fungal colonies were transferred to a new plate containing SNM agar by the use of a sterile toothpick, and ordered as shown in **Figure 2(a)**. Preliminarily, the microbial strains were named as LM (standing for *La Marquesa*) followed by a number which indicated the order of culturing.

2.4. Screening for Their Capacity to Grow on Cellulose and/or Hemicellulose

For testing their ability to grow on cellulose (CM-cellulose, Sigma) or hemicellulose (xylan from beechwood, Sigma) as the sole carbon source, each different fungal colony was transferred to a slant agar tube containing (per liter): 10 g of the polysaccharide, 2 g peptone, 5 g NaCl, 0.3 g K_2HPO_4, 0.15 g bromothymol blue, and 10 g agar-agar at a pH value of 7.0. This culture media was modified from the formulation reported by Hugh and Leifson [22], and glucose was employed as a positive control. Based on the formation of mycelium and on the color change of bromothymol blue, it was determined if the fungal strain was capable of growing on the carbon source or not.

2.5. Preservation of Fungi Selected for Their Ability to Grow on the Polysaccharides of Interest

Isolated strains were grown on SNM agar and incubated at 29˚C until the mycelium covered the Petri dish completely. To avoid desiccation, the edges of the dish were wrapped with a plastic film. Under sterile conditions, spores were harvested by soaking and rinsing the culture with 1 or 2 mL of 0.01% (v/v) Tween 80-water. The spore suspension derived from this was filtered (Whatman 40) to withdraw remaining mycelium, and 200 µL were reinoculated to 125-mL Erlenmeyer flasks containing 30 mL of solid SNM. As described above, the same procedure was followed for collecting spores except for the addition of 10 mL of 0.01% (v/v) Tween 80-water that were stirred with the aid of a magnetic bar (Thermolyne Cimarec 1 magnetic stirrer model S46415, 15 min at 600 rpm). After filtration, the suspension was centrifuged (10 min at 10,000 rpm), eliminating the supernatant and distributing 1 mL aliquots of spores in 1.5-ml sterile vials, which were kept at 4˚C until further use. Spore concentration was determined with a Neubauer chamber (Bright-Line hemacytometer, Hausser Scientific).

2.6. Morphological Characterization of the Strain Exhibiting the Fastest Growth on the Selected Polysaccharides

Due to its ability for growing on cellulose or hemicellulose as sole carbon sources and the relatively short time required to do so, strain LM-3 was selected for further studies. The first studies that were carried out included macroscopic and microscopic observation of the selected fungal strain after grown on solid Czapek agar, which was selected for culturing based on the colonies appearance. Czapek solid medium (per liter) consists of the following ingredients: 3.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 30.0 g sucrose, and 15.0 g agar [23]. Macroscopic features included colonial morphology, color, growth, and texture; meanwhile microstructures were ana-

lyzed with the 10 and 40x objectives of a binocular light microscope (Motic® Ba410) after following a slide culture procedure [24]. Derived from these observations, it was determined that the recently isolated fungal strain belonged to the genus *Penicillium*, therefore, for comparison purposes, a strain of *P. chrysogenum* (CFQ-H-46), purchased from the Microbial Collection of *Facultad de Química* (U.N.A.M.), was employed in subsequent experiments.

2.7. DNA Isolation from the Fungal Strains

250-mL Erlenmeyer flasks containing 50 mL of liquid SNM were inoculated with approximately 1 cm^2 of SNM agar densely covered with fungal mycelium and finely cut into very small pieces with the aid of a sterile scalpel. In order to know when the stationary phase took place, culture kinetics were performed, determining growth values by dry weight every 24 h, except for the time point monitored at 12 h. Once the microorganisms reached the stationary phase, fungal pellets were collected by filtration (Whatman 40), washed twice with 0.85% (w/v) NaCl, soaked with cold acetone, and left overnight for drying, inside a fume hood. The dried mycelium was grinded with a pestle over a mortar, adding dry ice five times, and the resulting powder was collected for storage at −20˚C until use. For DNA isolation, 0.1 g of the fungal powder was transferred to a 1.5-mL plastic vial sterile tube. 500 µL of lysis solution (50 mM Tris-HCl pH 7.1, 50 Mm EDTA pH 8.0, 3% of SDS, and 1% of 2-mercaptoethanol) were added, and the tube was vigorously stirred for 1 min with vortex. Followed to the addition of 20 µl of lysozyme (10 mg mL^{-1}), the mixture was incubated at 37˚C for 30 min, and after adding 35 µL of proteinase K (20 mg mL^{-1}) it was incubated at the same temperature for 1 h with rotary agitation (700 rpm). Next, 500 µL of chloroform were mixed, and the tube was vigorously stirred with vortex prior to centrifugation at 12,500 rpm for 15 min. After centrifuging, the aqueous phase (upper one) was recovered and transferred to a new 1.5-mL vial tube. 2.2 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate pH 8.0 were added, and the mixture was stored at −20°C at least for an hour to allow DNA to precipitate. After precipitation, the tube was centrifuged for 30 min at 4˚C and 12,500 rpm. Then, the supernatant was discarded, and the pellet, containing DNA, was washed with 500 µL of cold ethanol (70% v/v). Once again, the mixture was centrifuged for 10 min under the same conditions, ethanol was discarded, and the resulting pellet was air dried. Finally, the pellet was resuspended with 100 µL of ultrapure water and kept at −20˚C until use.

2.8. Agarose Gel Electrophoresis and Polymerase Chain Reaction

Prior to the polymerase chain reaction (PCR), the isolated DNAs, derived from the previous step, were examined for integrity and quantified by electrophoresis in agarose gel 1.0% (w/V), using 1X TAE buffer (80 V for 120 min), and stained with ethidium bromide. For all gel electrophoresis, GeneRuler 100 bp DNA ladder (Thermoscientific) served as standard for molecular weights.

The target corresponded to the internal transcribed spacer (ITS) of the ribosomal

DNA (rDNA) region, so universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were employed as forward and reverse, respectively [25]. Amplification was performed by PCR in a mixture containing the following reactives: buffer for polymerase 1X, 8 mM dNTPs, 2.5 mM $MgCl_2$, 2.5 mM of each primer, dimethyl sulfoxide (DMSO) 1X, and 12.5 U of Taq polymerase (HotStarTaq Master Mix Kit, Qiagen), up to a final volume of 100 µL. A thermocycler (Techne TC-512, Bibby Scientific Limited) was used with the following reaction program: 1) initial denaturation at 94˚C for 2 min, 2) 40 cycles of 94˚C (1 min), 50˚C (1 min), and 72˚C (2 min), and 3) a final extension at 72˚C during 5 min. The resulting amplicon was resolved by agarose gel electrophoresis under the conditions described above and visualized under UV light (Bio-Rad Gel Doc XR System). The observed bands were cut from the gel with a scalpel, and the PCR products were purified using the AxyPrep DNA gel extraction kit (Axygen) and following the specifications provided by the manufacturer.

2.9. Molecular Characterization of the Fungal Strain

The sequencing reaction of the products from the previous step was carried out with BigDye Terminator v3.1 according to the manufacturer instructions (Applied Biosystems). The purified samples were resuspended with 12 µL of formamide and resolved with a 3500XL capillary sequencer (24 capillaries). The derived sequences were compared in the GenBank database of the National Center for Biotechnology Information (NCBI), using the Basic Local Alignment Search Tool (BLAST) program [26] with the algorithm set to "blastn" (nucleotide-nucleotide BLAST).

2.10. SSF Using Pine Needles as Substrate

5.0 g of grinded pine needles of the fraction derived from sieve number 35 were transferred to Erlenmeyer flasks of 125-mL capacity, so the volume relationship between solid substrate and headspace was 0.2. Flasks were covered with cotton plugs, and sterilization of pine needles was accomplished by autoclaving during 30 min at 121˚C and 15 psi. Liquid SNM was added in order to attain a 60% moisture content of the solid substrate, and a fungal suspension of strain LM-3 or *P. chrysogenum* was inoculated at a concentration of 10^7 spores per gram of dry pine needles. Before incubation, solid state cultures were thoroughly mixed for attaining a higher degree of uniformity and then, kept at 29˚C under static conditions.

As controls, in order to determine if pine needles were employed for growth by the fungal strain labeled as LM-3, two variations of the process were tested. In the first case sterile distilled water was employed instead of the SNM solution; whereas, in the second control, pine needles were substituted by an inert matrix of commercial inorganic material (*Agrolita® Perlita*, *Minerales Expandidos, S.A. de C.V.*), mainly composed by silica (75.3%) and alumina (13.9%). For removal of impurities, *Agrolita®* was washed once with tap water and boiled twice in distilled water. Drying of the substrate was carried out overnight at 80˚C. After grinding with pestle and mortar, size fractionation was accomplished by sieving, as described for pine needles. Particles retained with sieve 35,

analogous in size to the fraction tested of lignocellulosic material were employed for SSF. The same relationship between solid substrate and headspace was maintained; therefore 7.5 g of *Agrolita®* were transferred to 125-mL Erlenmeyer flasks. The same procedure as for pine needles was followed for fungal cultures supported on the inorganic matrix for SSF.

2.11. Monitoring of the SSF Process

Fungal growth was followed by direct observation, using a stereo microscope (Stemi DV4) with a magnification power of 8x to 32x and by measuring the production of spores. Spore production was estimated by taking a 2 g sample of the solid culture and suspending it in Tween 80-water (0.01%, v/v) in a 1:10 proportion. After thoroughly mixing for 15 min, a 1-mL sample of the suspension was diluted in sterile distilled water, and the spore concentration was determined with a Neubauer chamber (Bright-Line hemacytometer, Hausser Scientific) as described above. On the other hand, C:N (carbon:nitrogen) ratios of the pine needles and the inoculated solid substrate at the initial and final time points were determined by elemental analysis. For this, the sample was weighed on a microbalance (Mettler Toledo model XP6) and analyzed at a combustion temperature of 1150˚C and a reduction temperature of 850˚C in an elemental analyzer (Elementar model vario MICRO cube). C:N ratios were only determined for cultures with LM-3 and *P. chrysogenum*, grown on pine needles supplemented with SNM, as the controls tested did not represent a solid basis for comparing this parameter for the cases in which fungi were grown on pine needles as the solid substrate. The content of lignin and holocellulose (cellulose plus hemicelluloses) was determined as described above [20] at the sixth day of the process. By this, it could be known whether the breakdown of polysaccharides took place or not. Finally, variation of pH was determined by taking a 1 g sample of the solid culture and suspending it in distilled water in a 1:10 proportion. After thoroughly mixing for 5 min, the pH value was measured with a potentiometer (Φ340 pH/Temp meter, Beckman) and after stirring once again for 5 min, the pH was registered again to verify that there were no changes in its premeasured value.

2.12. Statistical Analysis

All statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc.). For spore production, results were validated by a one-way analysis of variance (ANOVA) test, subsequently followed by a *post hoc* multiple comparison analysis (Tukey test). Subsequently, the production of spores was depicted by a box and whiskers plot in order to graphically show the differences observed between cultures under different conditions.

3. Results

3.1. Forest Plantation Characterization

After comparing the collected material obtained from the pine trees present in the for-

est plantation with the catalogs from MEXU, they were identified as *Pinus pseudostrobus* Lindl, a species considered to be native to Mexico, Guatemala, and Honduras [27]. Its wood possesses a good quality for diverse uses, such as sawmilling for lumber, construction, pulping for the paper industry, and even handcrafts. The characterized area presented a mean density of 130 trees per hectare, with a normal average diameter of 51.2 cm. A value of 45.8 Mg per hectare was estimated for the carbon stock accumulated in the dead biomass of this plantation, which lies in accordance to the reports by the Intergovernmental Panel on Climate Change (IPCC) [28] for litter of coniferous forests, ranging from 10 to 48 Mg of C ha^{-1}. The calculated value pointed out an excess of the stored carbon, so the withdrawal of a small fraction represented by pine needles has no stressing effect on the environment. Up to now, litter collected from this specific pine tree has received little or no attention, rather than being employed for starting controlled fires. For this scale of study, the production of plant litter and its decay are mostly influenced by dasometric features rather than by environmental factors.

3.2. Pine needles Characterization

As pine needles were collected during the dry season, their moisture content was determined to be less than 5.0%, so no drying process was required in order to avoid undesired microbial growth. After size reduction and fractionation the following proportions were found for three independent batches of 100 g of the lignocellulosic substrate: sieve 18 (18.1% ± 0.5%), sieve 35 (56.2% ± 3.1 %), and fine powders (25.6% ± 1.2 %). As the particles retained with sieve 35 represented the major fraction, it was selected for characterization and further use as substrate for SSF. The content of lignin, holocellulose, and extractives of this fraction expressed as percentage of dry matter was 47.4%, 27.0%, and 22.1%, respectively. It was determined that pine needles exhibited a high percentage of lignin (~50%), a finding expected due to their hardness, that might represent a potential hindrance for their use in SSF.

3.3. Fungi Screening

The ability of the strains, recently isolated from forest soil, to grow on cellulose or hemicellulose as the sole carbon source is presented in **Table 1**. Dense mycelium was observed with glucose as carbon source, which represented the positive control. However, notorious differences were found when the sixteen fungal strains were subjected to the polysaccharides of interest. Strain LM-3 followed by LM-8 exhibited the desired features, as both fungi were capable of growing on cellulose and hemicellulose as sole carbon sources. The presence of abundant mycelium and a shift from green to yellow of the culture medium, both indicated their capacity to metabolize the polysaccharides. However, in the specific case of LM-3, only 24 h were required for consuming either cellulose or hemicellulose, therefore, this strain was selected for evaluating it in SSF with pine needles as substrate. As a negative control, medium without carbon source was employed, in which no changes were detected at all.

Table 1. Screening for the ability to grow on selected polysaccharides of fungal strains isolated from *La Marquesa* National Park. Symbols: "++" represents dense mycelium and a clear color shift from green to yellow, "+" means scarce mycelium and a mild change in color (from green to light yellow), and "–" stands for no mycelium nor change in color.

Strain	Glucose	Cellulose	Hemicelllulose
LM-1	++	+	+
LM-2	++	++	+
LM-3	++	++	++
LM-4	++	+	+
LM-5	++	+	++
LM-6	++	–	–
LM-7	++	–	–
LM-8	++	++	++
LM-9	++	+	–
LM-10	++	–	–
LM-11	++	–	+
LM-12	++	++	–
LM-13	++	–	–
LM-14	++	–	+
LM-15	++	–	–
LM-16	++	+	+

3.4. LM-3 Morphological and Molecular Characterization

Macroscopic features of the fungal strain LM-3 grown on Czapek agar medium included a rapid growth of dark green colonies with granular texture and a white border (**Figure 2(b)**). The color of the back side of the colony was orange with a yellow border (**Figure 2(c)**). Microscopic characteristics can be observed in **Figure 2(d)**, highlighting the septate mycelium and the paintbrush-like structures composed of metulae, flask-shaped phialides and conidia, all typically found in the Aspergillaceae family. After carefully following illustrated guides and keys to various genera [23] [29] [30], based on the morphological features, it was determined that the isolated fungus belonged to the genus *Penicillium*.

Furthermore, the PCR products corresponded to the ITS region of rDNA which ranged between a molecular weight of 500 and 600 bp (**Figure 3**). For strain LM-3, the obtained sequence, excluding both primers, exhibited a length of 482 bp and was submitted to GenBank, which provided the following accession number: **KX499485**. The comparative analysis of the complete sequence was performed with BLAST program [26]. Hits showed an overall similarity value of 97% - 99% to several *Penicillium* species, most of which lacked an assigned specific epithet, and all scores presented an E-value of zero. These findings strengthened the results derived from the morphological

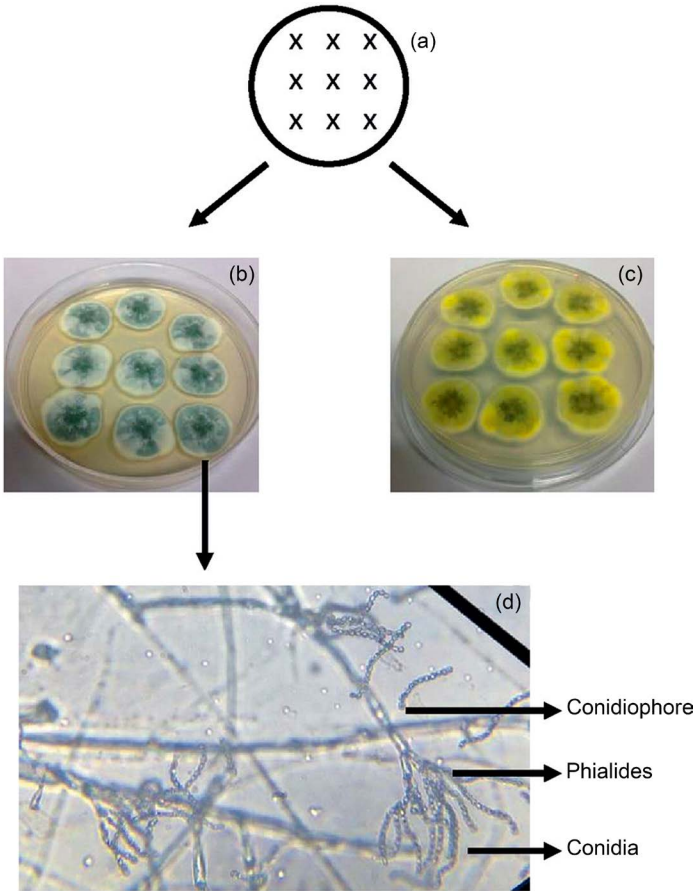


Figure 2. Transference scheme of fungi into Petri plate in order to obtain monosporic cultures (a), front (b) and back (c) views of fungal colonies, and microscopic characteristics at 40x (d).

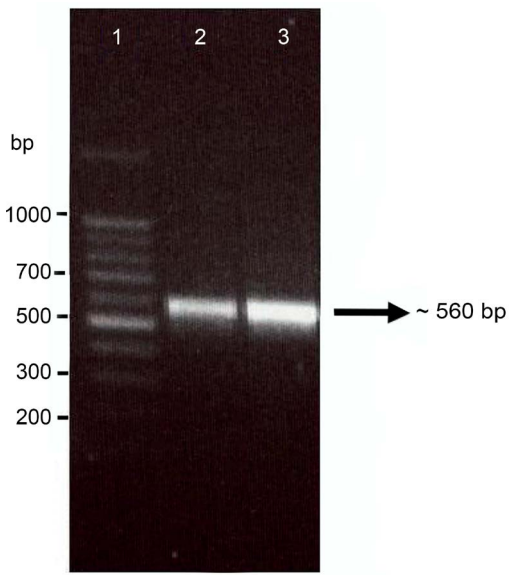


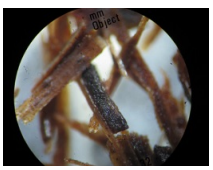
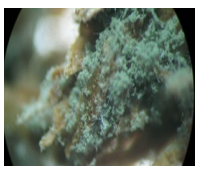
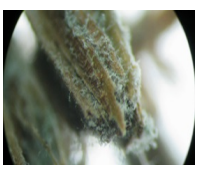
Figure 3. Agarose gel electrophoresis for visualizing the PCR product. Lane 1 represents the molecular weight marker (GeneRuler 100 bp DNA ladder, Thermo Scientific), lane 2 corresponds to the amplified target of the isolated fungal strain, and lane 3, to the product of *P. chrysogenum.*

characterization. Nonetheless, even though six *Penicillium* species (*P. ochrochloron*, *P. pulvillorum*, *P. subrubescens*, *P. rolfsii*, *P. janthinellum*, and *P. simplicissimum*) were displayed on the BLAST list of results, it is still not conclusive and might even be risky, to assign a specific epithet to the isolated fungal strain. As stated by some authors [31] [32], in the particular case of *Penicillium*, the ITS region is not variable enough for distinguishing all closely related species, making identifications of this genus, with BLAST, very tricky. Therefore, it was decided to leave the name of the isolated fungus as *Penicillium* sp. LM-3 in order to avoid uncertain nomenclature and misleading confusion, both negative aspects frequently found in *Aspergilli* and *Penicillia* [33]. On the other hand, in the case of the purchased fungal strain, the sequence analysis showed a 100% identity to *P. chrysogenum* with an E-value of zero, confirming that the method chosen for the molecular characterization of both fungal species was appropriate. However, further studies will be conducted in order to identify *Penicillium* sp. LM-3 at the species category or assign a new species to this fungus. In addition, computational approaches focused on constructing phylogenetic trees will be followed in the near future.

3.5. Solid State Fermentation

When the recently isolated fungal strain was compared to *P. chrysogenum*, also grown on pine needles, important differences were found (Table 2). A much higher production of spores was estimated from cultures in which *Penicillium* sp. LM-3 was tested, a result supported by the observations with the stereo microscope, in which, pine needles were covered with green mycelia. The amount of spores attained with this fungal strain was almost 70-fold with respect to the initial concentration. In the case of *P. chrysogenum*, white mycelium, barely covering the lignocellulosic material, was noticed;

Table 2. Comparison of strain LM-3, belonging to the genus *Penicillium*, with a purchased strain of the same genus (*P. chrysogenum* CFQ-H-46), when both were grown on pine needles as substrate for SSF, supplemented with SN liquid medium.

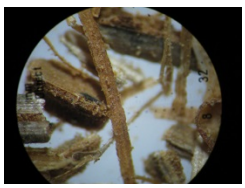
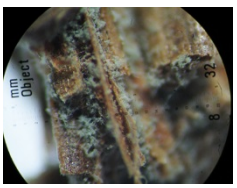
Parameter	*Penicillium* sp. LM-3		*P. chrysogenum*	
	Initial time point (t = 0)	End of the process (6 days)	Initial time point (t = 0)	End of the process (6 days)
Spores g^{-1} pine needles	$1 \times 10^7 \pm 4.8 \times 10^5$	$7 \times 10^8 \pm 1.5 \times 10^7$	$1 \times 10^7 \pm 3.9 \times 10^5$	$5 \times 10^7 \pm 1.7 \times 10^6$
Microscopic observation (20x)				
C:N ratio	68.02:1	51.26:1	67.90:1	62.13:1
Lignin (%)	ND[1]	46.8	ND	47.7
Holocellulose(%)	ND	19.5	ND	25.4
Extractives (%)	ND	27.6	ND	20.1
pH value	6.01 ± 0.07	5.04 ± 0.05	6.01 ± 0.08	5.72 ± 0.04

[1]ND refers to "not determined", as the composition was previously done for pine needles.

therefore, much lesser spores were quantified, in contrast to *Penicillium* sp. LM-3. This might be an indicative of the better ability of the strain isolated from forest soil to grow on plant litter. The latter was strengthened by the carbon to nitrogen ratio, which suffered a larger decrease in the case of the isolated fungus, indicating a higher growth of this strain when cultured on pine needles. C:N of pine needles was determined to be 88:1, a value that correlated to those reported on several web pages of common use for composting. After supplementation with SN medium and inoculation with fungal spores, the C:N ratio of the lignocellulosic material was lowered approximately to 68:1 for both cultures at the initial time of the process, corresponding to a carbon and nitrogen content in percentage (%) of 48.25 and 0.71, respectively. After the sixth day of the bioprocess, both fungal strains exhibited a decrease on one hand, and an increase on the other, in the C and N contents, respectively. In the case of *Penicillium* sp. LM-3 the carbon content was lowered to 40.3% and nitrogen increased up to 0.79%; meanwhile for *P. chrysogenum*, C and N reached a content of 46.6 and 0.75%, respectively. This finding pointed out that carbon, from supplemented glucose and pine needles, is most probably being used as energy source, and that nitrogen, from the SNL culture medium, is destined to building biomass material. However, a considerable difference of more than ten units in the C to N ratios for each case was calculated at the final time of the process, once more, indicating a better capacity of the recently isolated fungal strain to grow on pine needles. This statement was corroborated by the chemical analysis of the pine needles after the final time of the process. The lignocellulosic material derived from cultures of *Penicillium* sp. LM-3 exhibited no modification of the lignin content, while holocellulose and extractives, decreased 7 and suffered an increment of 5 percentage points, respectively. This tendency was not found in pine needles analyzed from *P. chrysogenum* cultures, in which the lignin was elevated not significantly, but contents of holocellulose and extractives were both lowered by approximately 2 percentage units. These results proved that the newly isolated microorganism was capable of the breakdown of polysaccharides in the form of holocellulose, while no considerable degradation was registered with the commercial fungal strain. Finally, variation in pH showed that *Penicillium* sp. LM-3 reached a value of approximately 5.0, while *P. chrysogenum* mildly dropped to 5.6. The latter might correlate again with the ability of each microorganism to utilize the lignocellulosic material as a carbon source, with the associated acidification of the medium.

On the other hand, several differences were perceived between solid cultures of *Penicillium* sp. LM-3 with pine needles as substrate without adding SNM and with the siliceous material as support supplemented with liquid medium (Table 3). A significant amount of spores were estimated when using the lignocellulosic material even though only distilled water was added at the initial time of the process. This finding pointed out at the ability of the fungus to grow on pine needles as the only carbon source, which in turn coincided with the natural habitat of forest soil microorganisms whose nitrogen availability is very low. However, compared to the case in which SNM was supplemented to the culture, the amount of spores produced was significantly lower,

Table 3. Solid state cultures of *Penicillium* sp. LM-3 grown on pine needles as substrate without addition of SN liquid medium, compared to a fermentation process supported on an inert material supplemented with SNM.

Parameter	Cultures grown on the lignocellulosic material		Cultures supported on silica and alumina	
	Initial time point (t = 0)	End of the process (6 days)	Initial time point (t = 0)	End of the process (6 days)
Spores g^{-1} solid material	$1 \times 10^7 \pm 5.1 \times 10^5$	$1.06 \times 10^8 \pm 9.7 \times 10^6$	$1 \times 10^7 \pm 6.2 \times 10^5$	$2.07 \times 10^7 \pm 1.6 \times 10^6$
Microscopic observation (20x)				
Lignin (%)	ND[1]	48.1	NC[2]	NC
Holocellulose (%)	ND	22.7	NC	NC
Extractives (%)	ND	24.4	NC	NC
pH value	5.98 ± 0.12	5.26 ± 0.08	5.99 ± 0.08	7.12 ± 0.05

[1]ND refers to "not determined", as the composition was previously done for pine needles; [2]NC stands for "not comparable", as the support exhibits none of the components determined by this method [20].

thus indicating the need of the microorganism for more readily metabolized carbon sources, such as glucose, and of the nitrogen provided by yeast extract. Interestingly, in this case the quantity of spores estimated was almost 2-fold higher than the found in cultures of *P. chrysogenum* with supplemented SNM, in spite that no notorious differences were observed with the stereo microscope. These data emphasized the convenient use of *Penicillium* sp. LM-3 as a better candidate for the implementation of a process based on the biodegradation of pine needles. In contrast, cultures of this fungal strain supported on the inert matrix of silica-alumina, showed a small increase in the spore content. This finding was also corroborated with the stereo microscope, as almost no mycelium was observed on cultures with the siliceous material. Even though liquid SNM was supplemented at the beginning of the process, apparently it was not sufficient enough to support the growth of the fungus over time.

Finally, variation of pH values showed two distinct profiles: an increase of more than one unit was registered in cultures grown on silica-alumina in comparison to a mild acidification in the case of pine needles employed as solid substrate. It is unclear the cause of the increment in the pH, but probably the exhaustion of glucose and the lysis of cells might account for it. On the other side, decrease in pH might be a consequence of metabolized sugars, probably derived from the breakdown of the lignocellulosic material. The latter reinforced the idea of the utilization of pine needles as carbon source by *Penicillium* sp. LM-3, which as shown above, when cultured with the addition of SN medium, thus containing a nitrogen source as well, exhibited a higher growth reflected by spore production.

3.6. Statistics

After performing the statistical analysis, the ANOVA test showed significant differenc-

es for the spore production, with a calculated F of 134.56 ($\alpha = 0.01$, and degrees of freedom $\nu 1 = 3$ and $\nu 2 = 19$). The *post hoc* Tukey test pointed that the significant difference was due to the condition in which the strain tested was *Penicillium* sp. LM-3 supplemented with SNM. Finally, with the box and whiskers plot for the production of spores (Figure 4) the differences described earlier and the significance, marked with asterisks (*), of this condition compared to the controls were readily observed. Therefore, the use of the recently isolated fungal strain grown on pine needles and supplemented with SNM is strongly recommended for this solid state fermentation novel process.

4. Discussion and Conclusions

Due to their hardness, pine needles are considered to be useless or of low-value for commercial applications. In spite of the latter, studies have been conducted that have proven their use as lignocellulose sources or adsorbing material. Few reports have been focused on their degradation at laboratory scale, with basidiomycete species of the following genera: *Gymnopus* [34], *Marasmius* [35], *Mycena* [34] [35] [36], and *Stereum* [37], as the biological model. In contrast to the latter studies, timing of the process carried out by *Penicillium* sp. LM-3 was considerably shorter, a finding that makes this fungal strain more attractive. Even when it was compared to a commercial strain of the same genus, its ability to grow on pine needles was more than 10-fold superior. In the very close future, research will be conducted with two different aims: 1) from a taxonomical approach, in order to identify the species of this fungus, and 2) from a biotechnological point of view, for the production of compounds with high commercial

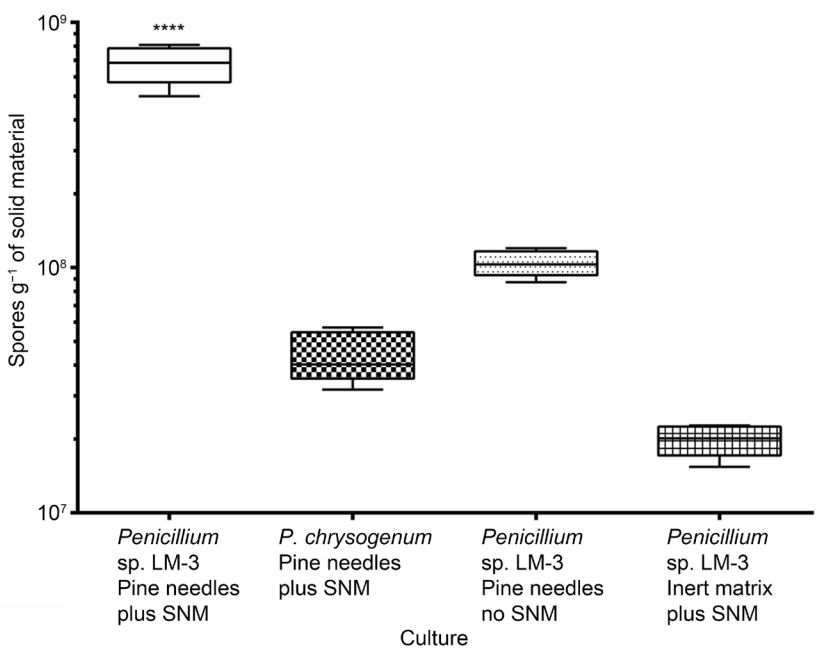


Figure 4. Box and whiskers plot for the spore production estimated for cultures with different parameters.

value such as primary and secondary metabolites, including cell-wall degrading enzymes (cellulases, xylanases, pectinases, and chitinases), which might include the manipulation of the process conditions for maximizing titers. From the present work, it can be concluded that a bioprocess based on the degradation of pine needles was developed at laboratory scale. This breakdown was carried out by a novel fungal strain characterized as a *Penicillium* species, which was isolated from a pine tree plantation located at *La Marquesa* National Park. *Penicillium* sp. LM-3 was selected, based on its desirable capacity for growing rapidly on cellulose and hemicellulose as carbon sources, and was further tested for SSF. Growing the fungus on the solid substrate yielded a maximum production of fungal spores after the sixth day, representing a less time consuming process compared to cultures of basidiomycetes [34] [35] [36] [37] and a more efficient one with respect to a different *Penicillium* strain (*P. chrysogenum*).

In addition to other ecosystem services, such as support, regulation, and recreation, the forest plantation of this study represents a very small area with an enormous potential for supplying bioresources. The provisioning services of this forest were destined for solid state fermentation with pine needles as the raw material and *Penicillium* sp. LM-3 as the genetic resource that constituted the biological model. It is believed that the withdrawal of a minimal fraction of the lignocellulosic material might even contribute for the prevention of fires, without affecting soil formation, so the process described in this work exhibits an eco-friendly advantage. There are still other fungal strains to be tested, such as LM-8, which showed a notable ability to grow on cellulose and hemicellulose as carbon sources. Furthermore, synergistic effects for biodegradation of lignocellulosics, obtained from mixed cultures, will also be evaluated, which might also include the action of soil bacteria. Mexico is the country with the highest diversity of pine trees [27] [38] and coniferous forests are widely spread throughout its territory, both of which represent an unlimited supply of novel resources still awaiting to be discovered and rationally exploited.

Plant litter accounts for an important amount of lignocellulosic material, whose biodegradation has been extensively studied. This paper represents the multidisciplinary work of areas such as forest science, chemistry, microbiology, molecular biology, and fermentation technology, for the implementation of a process focused on the utilization of biological resources obtained from a *Pinus pseudostrobus* plantation. In this case, these resources represented the two basic constituents for a solid state fermentation process: 1) plant litter itself, in the form of pine needles as the lignocellulose source, and 2) the soil microbiota, particularly a fungal strain identified as *Penicillium* sp. LM-3, which exhibited a promising capacity for spore production and holocellulose breakdown. This research represents the first report on the solid state cultivation of a *Penicillium* species grown on pine needles and constitutes the starting point for developing further bioprocesses based on these two, of the many other, bioresources from the selected area.

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References

[1] Young, R.A. and Giese, R.L. (1990) Introduction to Forest Sciences. John Wiley & Sons, New York.

[2] Bärlocher, F. (2005) Leaf Mass Loss Estimated by Litter Bag Technique. In: Graça, M.A.S., Bärlocher, F. and Gessner, M.O., Eds., *Methods to Study Litter Decomposition, a Practical Guide*, Springer, Dordrecht, 37-42. https://doi.org/10.1007/1-4020-3466-0_6

[3] Bishop, J. and Landell-Mills, N. (2006) The Environmental Services of Forests (Los Servicios Ambientales de los Bosques). In: Pagiola, S., Bishop, J. and Landell-Mills, N., Eds., *Selling Environmental Services of Forests: Market-Based Mechanisms for Conservation and Development* (*La Venta de Servicios Ambientales Forestales: Mecanismos Basados en el Mercado para la Conservación y Desarrollo*), Instituto Nacional de Ecología-Secretaría de Medio Ambiente y Recursos Naturales (INE-SEMARNAT), Mexico, 47-74.

[4] Coleman, D.C., Crossley Jr., D.A. and Hendrix, P.F. (2004) Fundamentals of Soil Ecology. Elsevier Academic Press, Burlington, San Diego, London.

[5] Spurr, S.H. and Barnes, B.V. (1982) Forest Ecology (Ecología Forestal). A.G.T. Editor, S. A., Mexico.

[6] Armson, K.A. (1977) Forest Soils: Properties and Processes. University of Toronto Press, Toronto. https://doi.org/10.3138/9781442656338

[7] Waring, R.H. and Schlesinger, W.H. (1985) Forest Ecosystems: Concepts and Management. Academic Press Inc., Orlando, San Diego.

[8] Adl, S.M. (2003) The Ecology of Soil Decomposition. CABI Publishing, Wallingford, Cambridge. https://doi.org/10.1079/9780851996615.0000

[9] Berg, B. and McClaugherty, C. (2008) Plant Litter: Decomposition, Humus Formation, Carbon Sequestration. Springer-Verlag, Berlin Heidelberg. https://doi.org/10.1007/978-3-540-74923-3

[10] Swift, M.J., Heal, O.W. and Anderson, J.M. (1979) Decomposition in Terrestrial Ecosystem. Blackwell Scientific Publications, Oxford.

[11] Hättenschwiler, S., Tiunov, A.V. and Scheu, S. (2005) Biodiversity and Litter Decomposition in Terrestrial Ecosystems. *Annual Review of Ecology, Evolution, and Systematics*, **36**, 191-218. https://doi.org/10.1146/annurev.ecolsys.36.112904.151932

[12] Gernandt, D.S. and Pérez de la Rosa, J.A. (2014) Biodiversity of Pinophyta (Conifers) in Mexico [Biodiversidad de Pinophyta (Coníferas) en México]. *Revista Mexicana de*

Biodiversidad, **85**, S126-S133. https://doi.org/10.7550/rmb.32195

[13] Flores Mata, G., Jiménez López, L., Madrigal Sánchez, X., Moncayo Ruiz, F. and Takaki Takaki, F. (1971) Map and Description of the Types of Vegetation of the Mexican Republic [Mapa y Descripción de los Tipos de Vegetación de la Repúbica Mexicana]. SRH, Dirección de Agrología, Mexico.

[14] Antartis (2015) Highly Detailed Planet Earth. Mexico Elements Furnished by NASA. https://stockfresh.com/image/6510776/detailed-earth-mexico

[15] Google Maps (2015) https://www.google.com.mx/maps/@19.3146744,99.3933926,5503m/data=!3m1!1e3?hl=en

[16] Pandey, A., Soccol, C.R., Rodriguez-Leon, J.A. and Nigam, P. (2001) Solid-State Fermentation in Biotechnology-Fundamentals and Applications. Asiatech Publishers Inc., New Delhi.

[17] Krishna, C. (2005) Solid-State Fermentation Systems—An Overview. *Critical Reviews in Biotechnology*, **25**, 1-30. https://doi.org/10.1080/07388550590925383

[18] Lavelle, P. and Spain, A.V. (2005) Soil Ecology. Springer, Dordrecht.

[19] Brady, N.C. and Weil, R.R. (2008) The Nature and Properties of Soils. Pearson Prentice Hall, Columbus.

[20] Van Soest, P.J. (1963) Use of Detergents in the Analyses of Fibrous Feed. II. A Rapid Method for the Determination of Fiber and Lignin. *Journal of the Association of Official Agricultural Chemists*, **46**, 829-835.

[21] Zorn, H., Langhoff, S., Scheibner, M. and Berger, R.G. (2003) Cleavage of Carotene by Fungi. *Applied Microbiology and Biotechnology*, **62**, 331-336. https://doi.org/10.1007/s00253-003-1309-4

[22] Hugh, R. and Leifson, E. (1953) The Taxonomic Significance of Fermentative versus Oxidative Metabolism of Carbohydrates by Various Gram Negative Bacteria. *Journal of Bacteriology*, **66**, 24-26.

[23] Raper, K.B. and Thom, C. (1949) A Manual of the Penicillia. The Williams & Wilkins Company, Baltimore.

[24] Riddell, R.W. (1950) Permanent Stained Mycological Preparations Obtained by Slide Culture. *Mycologia*, **42**, 265-270. https://doi.org/10.2307/3755439

[25] White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis, M.A., Gelfand, D.H. and Sninsky, J.J., Eds., *PCR Protocols: A Guide to Methods and Applications*, Academic Press Inc., New York, 315-322. https://doi.org/10.1016/b978-0-12-372180-8.50042-1

[26] Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs. *Nucleic Acids Research*, **25**, 3389-3402. https://doi.org/10.1093/nar/25.17.3389

[27] Sáenz, R.J.T., Muñoz, F.H.J. and Rueda, S.A. (2011) Promissory Species of Temperate Climate for Commercial Forest Plantations in Michoacan [Especies Promisorias de Clima Templado para Plantaciones Forestales Comerciales en Michoacán]. Libro Técnico Núm. 10. SAGARPA-INIFAP-CIRPAC-CAMPO Experimental Uruapan, Uruapan.

[28] IPCC (2003) Good Practice Guidance for Land Use, Land-Use Change and Forestry. Penman, J., Gytarsky, M., Hiraishi, T., Krug, T., Kruger, D., Pipatti, R., Buendia, L., Miwa, K., Ngara, T., Tanabe, K. and Wagner, F., Eds., Institute for Global Environmental Strategies (IGES), Kanagawa.

[29] Barnett, H.L. and Hunter, B.B. (1987) Illustrated Genera of Imperfect Fungi. Macmillan

Publishing Co., New York, and Collier Macmillan, London.

[30] Domsch, K.H., Gams, W. and Anderson, T.-H. (2007) Compendium of Soil Fungi. IWH-Verlag, Eching.

[31] Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spoug,e J.L., Levesque, C.A., Chen, W. and Fungal Barcoding Consortium (2012) Nuclear Ribosomal Internal Transcribed Spacer (ITS) Region as a Universal DNA Barcode Marker for Fungi. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 6241-6246. https://doi.org/10.1073/pnas.1117018109

[32] Visagie, C.M., Houbraken, J., Frisvad, J.C., Hong, S.B., Klaassen, C.H., Perrone, G., Seifert, K.A., Varga, J., Yaguchi, T. and Samson, R.A. (2014) Identification and Nomenclature of the Genus *Penicillium*. *Studies in Mycology*, **78**, 343-371. https://doi.org/10.1016/j.simyco.2014.09.001

[33] Houbraken, J., de Vries, R.P. and Samson, R.A. (2014) Modern Taxonomy of Biotechnologically Important *Aspergillus* and *Penicillium* Species. *Advances in Applied Microbiology*, **86**, 199-249. https://doi.org/10.1016/B978-0-12-800262-9.00004-4

[34] Boberg, J.B., Finlay, R.D., Stenlid, J., Ekblad, A. and Lindahl, B.D. (2014) Nitrogen and Carbon Reallocation in Fungal Mycelia during Decomposition of Boreal Forest Litter. *PLoS ONE*, **9**, e92897. https://doi.org/10.1371/journal.pone.0092897

[35] Boberg, J.B., Nasholm, T., Finlay, R.D., Stenlid, J. and Lindahl, B.D. (2011) Nitrogen Availability Affects Saprotrophic Basidiomycetes Decomposing Pine Needles in a Long Term Laboratory Study. *Fungal Ecology*, **4**, 408-416. https://doi.org/10.1016/j.funeco.2011.03.004

[36] Boberg, J., Finlay, R.D., Stenlid, J., Näsholm, T. and Lindahl, B.D. (2008) Glucose and Ammonium Additions Affect Needle Decomposition and Carbon Allocation by the Litter Degrading Fungus Mycena epipterygia. *Soil Biology and Biochemistry*, **40**, 995-999. https://doi.org/10.1016/j.soilbio.2007.11.005

[37] Mouso, N., Diorio, L.A. and Forchiassin, F. (2003) Degradation of Pine Needles by *Stereum hirsutum*. *Revista Argentina de Microbiología*, **35**, 219-223.

[38] Martínez, M. (1948) The Mexican Pines [Los Pinos Mexicanos]. Ediciones Botas, Mexico.