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Characterization of three novel pigment-producing *Penicillium* strains isolated from the Mexican semi-desert

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Fungal pigments are used in diverse industries such as food, pharmaceuticals, textile, among others. The need of new organic pigments involves the search for new microbial sources. In this study, three fungal strains isolated from *Quercus* sp and *Larrea tridentata* were morphological, physiological and molecularly characterized. Different temperatures (8, 16, 20, 24 and 32°C) and pH (4, 6, 7, 8 and 10) levels were tested to determine the best conditions to produce a fungal pigment under submerged and solid state fermentation. The three strains were genotyped by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), random amplified polymorphic DNA (RAPD), inter-transcript spaces (ITS) and intergenic spaces (IGS) with the object to eliminate duplications. The strains were identified according to their morphology as *Penicillium purpurogenum* (GH2) and *Penicillium pinophilum* (EH2 and EH3). It was found that at submerged state fermentation to 24°C and pH 10, the three strains produced pigments, but *P. purpurogenum* GH2 strain produced the highest amount of pigments (1.25 U). According to the molecular analysis, it was found that all strains were different. This is to our knowledge the first report on characterization of fungal strains isolated from the Mexican semi-desert which have potential for pigment production.

Key words: *Penicillium purpurogenum, Penicillium pinophilum*, intergenic spaces (IGS), inter-transcript spaces (ITS), pigments, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), submerged and solid state fermentation.

INTRODUCTION

Pigments are molecules of great importance for industries such as food, cosmetics, pharmaceuticals and textiles. At the present, there is great interest in the use and consumption of natural compounds, due to health problems associated with some synthetic pigments, and some benefits associated with some natural pigments (Downham and Collins, 2000; Knecht and Humpf, 2006; Knecht, 2006; Yang, 2006). In nature, there are many

sources for pigment production, however, only a fraction of them are available in sufficient quantity to be used at industrial level (Duffosé et al., 2005; Mapari et al., 2005). Microorganisms are a source of natural pigments. In addition, these organisms can grow into different culture systems (submerged and solid) and can be genetically modified to increase productivity and quality of the produced pigments (Campoy et al., 2003; Yan et al., 2005). Among the microorganisms that produce pigments are: filamentous fungi *Monascus* spp., *Penicillium* spp., *Paecylomices* spp. (Blanc et al., 1994; Méndez-Zavala et al., 2007; Mapari et al., 2006), yeasts *Paffia rhodozyma* and microalgae *Porphyridium cruentum* and *Haematoccocus pluvialis* (Boussiba, 2000; Yoshimura et al., 2006).

Monascus spp. has been studied in detail for its production of more than six red-colored (rubropunctatine and monascorrubramine), oranges (rubropuctamine and monascorrubramine) and yellow (monascine and ankaflavine) pigments (Blanc et al., 1994; Méndez-Zavala et al., 2007; Mapari et al., 2006; Boussiba, 2000; Yoshimura et al., 2006; Carvalho et al., 2003) and some other metabolites recently found with characteristics of blue fluorescence isolated from rice fermented with this fungus (Huang et al., 2008). *Monascus* spp. had a great potential for the food industry (Carvalho et al., 2003) because its production of pigments is allowed in Asian countries. These pigments present a polyketide structure (Mapari et al., 2010), and represent a family of highly structurally diverse compounds, all produced via iterative decarboxylative condensations of starter and extender units, analogous to the biosynthesis of fatty acids (Robinson, 1991; Baerson and Rimando, 2007); an example is lovastatin (Staunton and Weissman, 2001) which have anti-cholesterol properties, natamycin (antibiotic) used as food additive. Angkak is widely used in meat products (chicken salt-baked meat products halogenated sauce), flavorings, beverages, wines, fruit jelly, cakes, pastries and candy and it is an excellent natural substitute colorant for the synthetic pigments such as tartrazine (Dikshit and Tallapragada, 2011, Feng et al., 2012), and riboflavin (vitamin B, a yellow pigment with permitted use in different countries) (Carbadlho et al., 2007). On the other hand, some pigments homologous to those of *Monascus* spp., by strains of *Penicillium* spp. had been reported (Mapari et al., 2006; Ogihara et al., 2001; Jiang et al., 2005). Some of these pigments are molecules known as PP-R (7-(2-Hidroxyethyl)-monascorubramine (Mapari et al., 2006). These pigments make the genus *Penicillium* a potential source of pigments with future industrial use. Another of the pigments isolated from *Penicillium purpurogenum* biomass in liquid cultures, is a series of compounds known as purpurogenone and its derivatives (hidroxipurpurogenone, glitter, among others) (Duffosé et al., 2005; Roberts and Thompson, 1971a, 1971b). It is reported that the pigment production by *P. purpurogenum* (GH2) is enhanced when the culture media have low molecular weight carbon sources (Cruz-Hernández et al., 2005). Red pigments produced by other fungal strains (*Monascus purpureus)* have been reported (Mapari et al., 2006). This work shows the characterization of three *Penicillium* spp. strains isolated from the Mexican semi-desert (Cruz-Hernández, et al., 2005), which produce red pigments. There is only one report about the metabolic activity of these fungal strains (Hernández-Rivera et al., 2008), which has not been characterized in a molecular way. The objectives of this work were: 1) to characterize taxonomically three fungal strains; 2) to evaluate the effect of pH, temperature and culture system on pigment production and 3) to determine the DNA fingerprinting of the three *Penicillium* spp. strains by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of random amplification of polymorphic DNA (RAPDs), internal transcribed spaces (ITS) and inter-genetic spaces (IGS) in order to eliminate duplications.

MATERIALS AND METHODS

Isolation and morphological characterization

The three fungal strains were obtained from the Food Research Department- Universidad Autonoma de Coahuila (DIA-UAdeC) collection. Two (EH2 and EH3) fungal strains were isolated from oak leaves (*Quercus* spp.) and another one (GH2) from creosote bush (*Larrea tridentata*) leaves by Cruz-Hernández et al. (2005). The fungal strains were propagated on potato-dextrose agar (PDA, BD BIOXON; 39.9 gL^{-1}) and purified by monosporic cultures using the same medium. The purified fungal cultures were stored at -20°C in a cryo-preservative solution of skim milk/glycerol and preserved at the University of Minho Portugal fungal collection. For morphological characterization, all fungal strains were grown on three different culture media: malt extract agar (MSA, Difco), potato dextrose agar-PDA (Bioxon) and Czapek-dox agar (sucrose 30g, $NaNO_3$ 2g, K_2HPO_4 1g, $MgSO_4$ 0.5g, KCl 0.5g, $FeSO_4$ 0.01g and agar 15g L^{-1} in distilled water) and incubated at 30°C between 5 to 7 days. After that, mycelium and spores were placed on slides and fungal morphological characteristics were evaluated using light microscopy (Leiden Model MC-319).

Effect of pH and temperature on pigment production

The effect of pH and temperature on pigment production of each fungal strain was determined using a Plan Puebla II treatment matrix (Table 1), where the 0 and 10 values in the treatments matrix were replaced by 4 and 10 for pH and 8 and 32°C for temperature. The pH effect was evaluated using Petri dishes that contained PDA solid medium (39 g/L^{-1}); in this case, five pH level were evaluated

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Abbreviations: PCR-RFLP, Polymerase chain reaction-restriction fragment length polymorphism; **RAPD,** random amplification of polymorphic DNA; **ITS,** internal transcribed spaces; **IGS,** inter-genetic spaces; **PDA,** potato-dextrose agar; **MSA,** malt extract agar; **SmF,** submerged fermentation; **SSF,** solid state fermentation; **AME,** agar-malt extract.

Table 1. Growth of three *Penicillium* strains (GH2, EH2 and EH3) at 5 pH and 5 temperature levels.

Temperature (°C)	pH	Strain growth		
		GH_2	EH_2	EH_3
8	6	-	-	-
16	4	+	+	+
16	6	+	+	+
16	8	-	-	-
20	7	+	+	+
24	6	+	+	+
24	8	+	+	+
24	10	+	+	+
32	8	+	+	+

+, Growth; -, no growth.

(4, 6, 7, 8 and 10) The pH level was adjusted with 0.1 M HCl or 0.5 M NaOH. The evaluated temperature conditions were 8, 16, 20, 24 and 32°C. The pH and temperature treatments were established under a randomized complete block design with three replications. Each Petri dish with a specific pH level was inoculated with $2x10^7$ fungal spores suspension and then incubated at the programed temperatures for each treatment. An exploration of absorbance under the UV-visible range (350 to 600 nm) was performed in order to know the absorbance wave maximum longitude of the extract with the pigments. This absorbance was 414 nm.

Pigment production under solid and submerged state fermentation

Pigment production was carried out under submerged (SmF) and solid state (SSF) fermentation. SSF was performed using agar-malt extract (AME) (33.6 gL^{-1}) and SmF was conducted in 250 mL Erlenmeyer flasks containing 50 mL of malt extract (DIFCO; 15.0 gL^{-1}). In both cases, pH was adjusted to 10. Besides, reactors were inoculated in the center with $2x10^7$ fungal spores suspension and incubated at 24°C for 14 days; this temperature was selected because the absorbance value was the highest for the extract containing the pigments. Erlenmeyer flasks under SmF were vortexes to 200 rpm. After 14 days of incubation, produced biomass was recovered from both SmF and SSF and Petri dishes with solid medium was treated as follow: medium was liquid and 60 mL of distilled water was added at 100°C, so pigment and biomass were removed from agar by filtering using Wathman paper No. 1. In both cases, biomass was dried in an oven for 24 h at 60°C, and then by a gravimetric procedure, biomass production was determined.

The extract from SmF was filtered using a vacuum and 0.45 µm millipore membrane. An aliquot of 0.1 mL of the filtered extract was diluted 1:10 with sterile distilled water. Then, this dilution was used to conduct an exploration of absorbance under the UV-visible range (350 to 600 nm). In all the cases, pigment yield determination was done using a spectrophotometer at 414 nm.

DNA isolation

All three fungal strains were grown in 250 ml Erlenmeyer flasks containing 50 ml of the malt extract culture medium at SmF; each flask was inoculated with $2x10^7$ fungal spores suspension and incubated for 7 days at 24°C and pH 10 with an agitation of 200 rpm to obtain biomass for DNA extraction. Fungal DNA isolation was performed by Graham et al. (1994) method. The DNA quality was determined using agarose gel electrophoresis. The gel was prepared with agarose 1% (Sigma-Aldrich, St. Louis, MO, USA) in Tris Borate EDTA (Sigma-Aldrich) (TBE 0.5%) with 0.5 µL/mL of ethidium bromide (EtBr) (Sigma-Aldrich). The running conditions were constant at 95 V for 40 min in TBE 0.5× buffer.

Random amplification of polymorphic DNA (RAPD)

The RAPD determinations used three different primers (A02) 5' TGC CGA GCT G 3', (A08) 5' GTG ACG TAG G 3' and (D06) 5' ACC TGA ACG C 3'; some of these primer have been previously used for characterization of fungal strains (Dupont et al., 1999; Cervelantti et al., 2002). 23 µL reaction mixture contained 1× PCR buffer (20 nm Tris, pH 8.4, 50 mM KCl) (Invitrogen, Carlsbad, CA, USA), 0.5 uL of dNTPs at 20 mM (Invitrogen), 2.0 mM $MgCl_2$ (Invitrogen), 0.5 µM of each primer (Invitrogen), and 0.1 U/µL of Taq DNA polymerase (Bioline, London, UK). PCR reaction was performed by placing 2 µL of fungal DNA into a centrifugation tube and then the PCR reagent was added. The DNA in the centrifugation tube was incubated in a PCR thermocycler model Px2 (Thermo Electron Corporation, Milford, MA, USA) under the following program: 94°C for 2 min; 35 cycles of 92°C for 1 min, an annealing temperature of 36°C for 1 min, 72°C for 2 min and a final extension at 72°C for 5 min. The amplified segments were visualized by agarose (1.0%) gel electrophoresis.

Internal transcribed spacer (ITS) and intergenic spaces (IGS) amplification

A 23 µL reaction mixture contained 1× PCR buffer (20 nm Tris, pH 8.4, 50 mM KCl) (Invitrogen, Carlsbad, CA, USA), 0.2 Mm dNTPs (Invitrogen), 2.0 mM $MgCl_2$ (Invitrogen), 0.5 µM of each primer (Invitrogen), and 0.1 U/µL of Taq DNA polymerase (Bioline, London, UK). PCR reaction was performed by spiking 2 µL of fungal DNA into a centrifugation tube and then the PCR reagent was added. The DNA in the centrifugation tube was incubated in a PCR thermocycler model Px2 (Thermo Electron Corporation, Milford, MA, USA) under the following program: 95°C for 5 min; 35 cycles of 94°C for 1 min, an annealing temperature of 54°C for IGS and cry, 50°C for ITS for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. The primer pair IGSF 5´CTG AAC GCC TCT AAG TCA G 3´

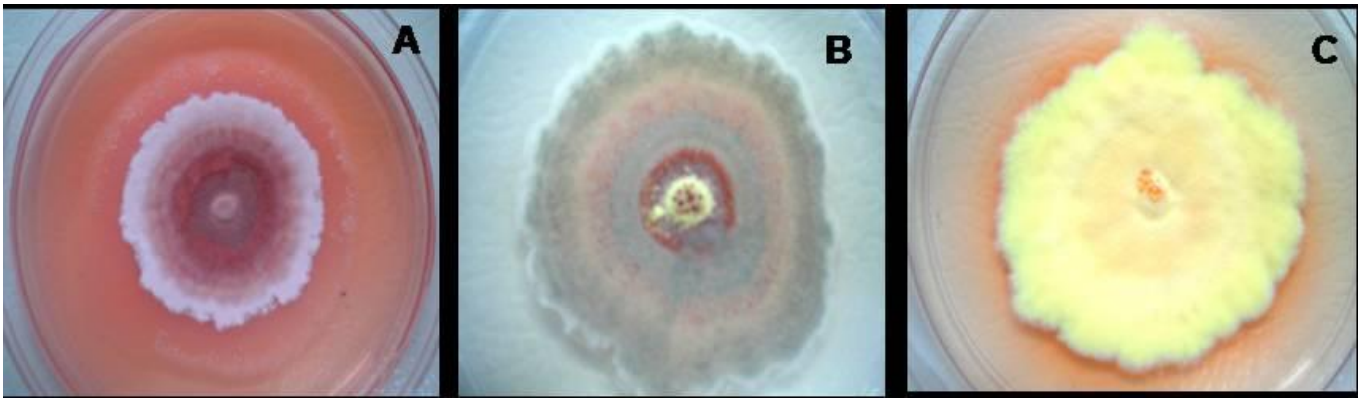

Figure 1. 14-day old growth of *Penicillium* sp, GH2 strain on three different culture mediums. A, Agar-malt extract (AME); B, potato-dextrose agar (PDA); C, Czapeck Dox medium.

and IGSR 5´AAT GAG CGA TTC GCA GTT TC 3´ was used to amplify the IGS region (Anderson and Stasovski, 1992). Then, the ITS4 5´TCC TCC GCT TAT TGA TAT GC 3´ and ITS5 5´GGA AGT AAA AGT CGT AAC AAG G 3´ primer pair was used to amplify the ITS region; these are universal fungal primers using for identifying of fungi (Hinrikson et al., 2005). Oligonucleotide primers were used as purified and as desalted specimen from Invitrogen. The amplified segments were visualized using agarose (1.50%) gel electrophoresis. The ITS and IGS amplified segments were digested with four restriction enzymes (Hind III, Eco RI, Xho I and Bam I-Hind III) as follow: 1 uL of amplified DNA was placed in microtube, and added 16.3 µl of sterile deionized distilled water, 2.0 µl of 10X buffer, 0.2 µl of bovine serum albumin (0.1%), and 0.5 µl (12 u/uL) of restriction enzyme. The tubes were mixed and centrifuged briefly and later incubated for 12 h at 37°C. The digested segments were separated using polyacrylamide (15.0%) gel electrophoresis; these polyacrilamide concentration has been reported suitable to separate DNA fragments lower than 500 bp (Laemmli, 1970) and gel were stained with silver nitrate following the methodology reported by Switzer et al. (1979). Patterns of the ITS and IGS digested fragments were coded as follows: Band present = 1, band absent = 0, no data = NA, and analyzed using the software S-PLUS -2000.

RESULTS AND DISCUSSION

Fungal morphological characterization

The pigment-producing fungal strains were identified microscopically as belonging to the *Penicillium* genus, according to their characteristics of structure and form of radial growth on solid culture media, identification was performed using the taxonomic keys for filamentous fungi (Gilmain, 1957) and characterized by the observation of pigment production under three different culture mediums and endorsed by Dr. Sophie Kozakiewics from the fungi culture collections of the University of Minho.

The *Penicillium* spp. GH2 strain, grew radially on PDA (Figure 1A), covering the whole dish (100 mm) after 12 days of growth. This strain produced pigments from orange-red to red-purple as it grew and this was more evident in the dish back although these pigments later spread throughout the whole culture medium. The GH2 strain mycelium was from yellow-orange to red in the center of the dish, with white edges and presented a green sporulation at 12 days of growth. On Czapek-dox medium (Figure 1B), fungal growth was slower compared to PDA medium, presenting a growth of 60 mm diameter after 14 days of culture, showing a red-orange pigment with little spread in the culture medium. On the AME medium (Figure 1C), the GH2 strain presented pigmentation on the back of the colony which spread as the GH2 strain grew; fungal mycelium was white with a red center of the colony after 12 days of culture. The *Penicillium* spp. EH2 strain (Figure 2A), showed similar colonial characteristics to GH2 strain on PDA, AEM and Czapek-dox media. At 12 days of growth, the EH2 strain showed a colonial diameter of 46 mm with a yellow mycelium and a pale green sporulation area. In addition, this strain produced an orange-red pigment on PDA medium.

Penicillium spp. EH3 strain (Figure 2B), had a radial growth very similar to the EH2 strain. The growth on the three culture media were similar to EH2, showing a radial growth with a diameter of 72 mm after 14 days of culture. On PDA medium, EH3 mycelium color was yellow with a white area in the center. Besides, this strain showed a green sporulation area. The pigment produced was orange to red which was diffused in the culture medium. It is reported a 70 mm radial growth after 14 days of culture of a *Penicillium* spp strain, which was characterized as *Monascus* pigment producer (Jiang et al., 2005). The growth velocity showed in this reported strain was similar to the growth velocity showed for the *Penicillium* strains evaluated in this study.

Morphology microscopic taxonomic identification

The GH2 strain was identified as *Penicillium purpurogenum*. The strain belongs to the symmetrical biverticillate group; it did not showed cleistothecium or sclerotium, but it showed single conidiophores with chains of conidia and it did not showed a coremium. Conidia were observed elliptical, globose and rough with rough and apiculate walls, it was observed as lanceolate phialides and from 3 to 5 phialides per metula. The EH2 and EH3 strains were

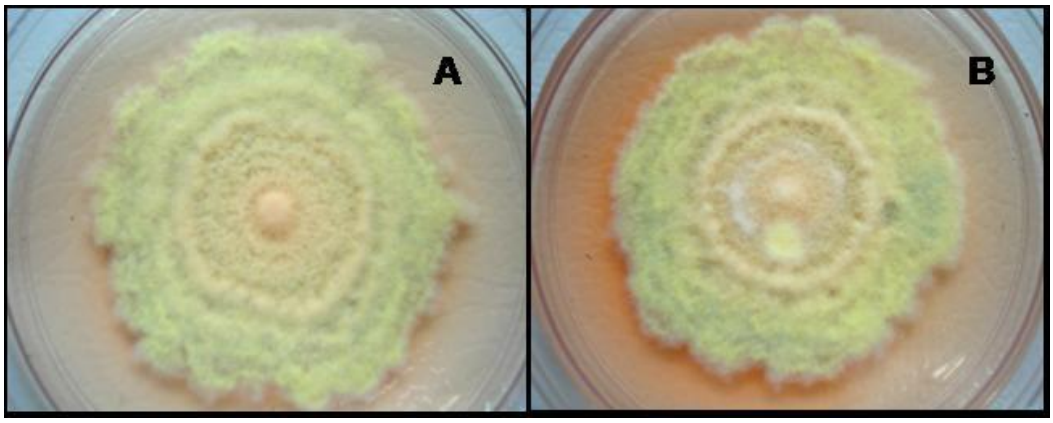

Figure 2. 14-day old growth of *Penicillium* sp, strain on PDA medium at 24°C / pH 7. A, EH2; B, EH3.

identified as *Penicillium pinophilum*. The strains did not show cleistothecium or sclerotium, it showed conidiophores without ramifications and forming coremium; apiculate conidia showed an ellipsoidal to fusiform shape with smooth wall; each metula showed 3 to 5 apiculate phialides.

Effect of pH and temperature on pigment production

Table 1 shows the growth of three fungal strains in all evaluated treatments. The fungal strains grew on all treatments except for 8°C/pH 6 and 16°C/pH 8 treatments, which show the mesophilic nature of fungi. At a temperature of 24°C and pH 10, the three strains showed the highest pigment production, however the pH value in each strain at 24°C made a difference in color between each strain to achieve different levels of pigment production, indicating a direct effect due to the initial pH value, however the combined effect of pH and temperature caused a direct effect on pigment production, probably as a result of environmental conditions from where the strains were isolated (semidesert).

The optimum pH for *Monascus* spp. pigment production was different from that of maximum biomass production, showing the best pigments production at pH 4.0 and the best biomass production at pH 6.5 (Carvalho et al., 2003). While some studies with a *Penicillium* spp. strains under submerged fermentation reported that the optimum pH for growth and pigment production was 9.0 and the optimum temperature for pigment production of 30°C (Gunasekaran and Poorniammal, 2008), a fact that was not found in this investigation and has been demonstrated that composition of culture medium affects directly *P. purpurogenum* growth and pigment production, fungal colony morphology varied dramatically from one culture medium to another. In addition, invasion velocity was also affected by culture medium composition (Méndez-Zavala et al., 2007). It is reported that pigment production is affected by substrate concentration on minimal culture medium, which reflects the nature of secondary metabolite of the pigments produced by *P. purpurogenum* strains, which can be produced as a defense mechanism (Hernández Rivera, 2006). Different authors suggest that microorganisms regulate the pH in the culture medium as an adaption at the different conditions to which they are exposed, and so be able to carry out their physiological and biochemical duties. It was reported that a *P. purpurogenum* strain presented on PDA medium, a maximum growth rate of 0.1046 mmh^{-1} at 26.6°C and pH 4.5; temperature value which coincides with that obtained in this study but pH value do not (Lafuente Castañeda, 2004).

Pigment production on solid and submerged state culture

The three *Penicillium* spp. strains were able to grow on the two fermentation systems at pH 10 and 24°C (Figure 3A). The EH2 strain under SmF produced the highest amount of biomass (0.172 $gbiomass.gflask^{-1}$), while the GH2 strain produced the highest amount of biomass (0.038 g biomass.g Petri $dish^{-1}$) on SSF. The pH and temperature values were those found as the best for pigment production in the last step, but these condition were not the best for biomass production in both fermentation systems. *P. purpurogenum* GH2 strain produced the highest amount of pigment (1.25U) at 24°C and pH 10, under SSF. The pigment production of this strain presented greater stability than that of *P. pinophilum* EH2 and EH3 strains (Figure 3B), because these strains produced pigment in a discontinuous or unstable way under SSF. These results are consistent with those reported (Méndez-Zavala et al., 2007) which showed that *P. purpurogenum* GH2 produced higher amount of extracellular pigments on malt extract than that produced on Czapek-dox, Hiroi or sucrose mediums. The GH2 strain produced pigment after 12 days of culture, reaching the maximum pigment production at 14 days of

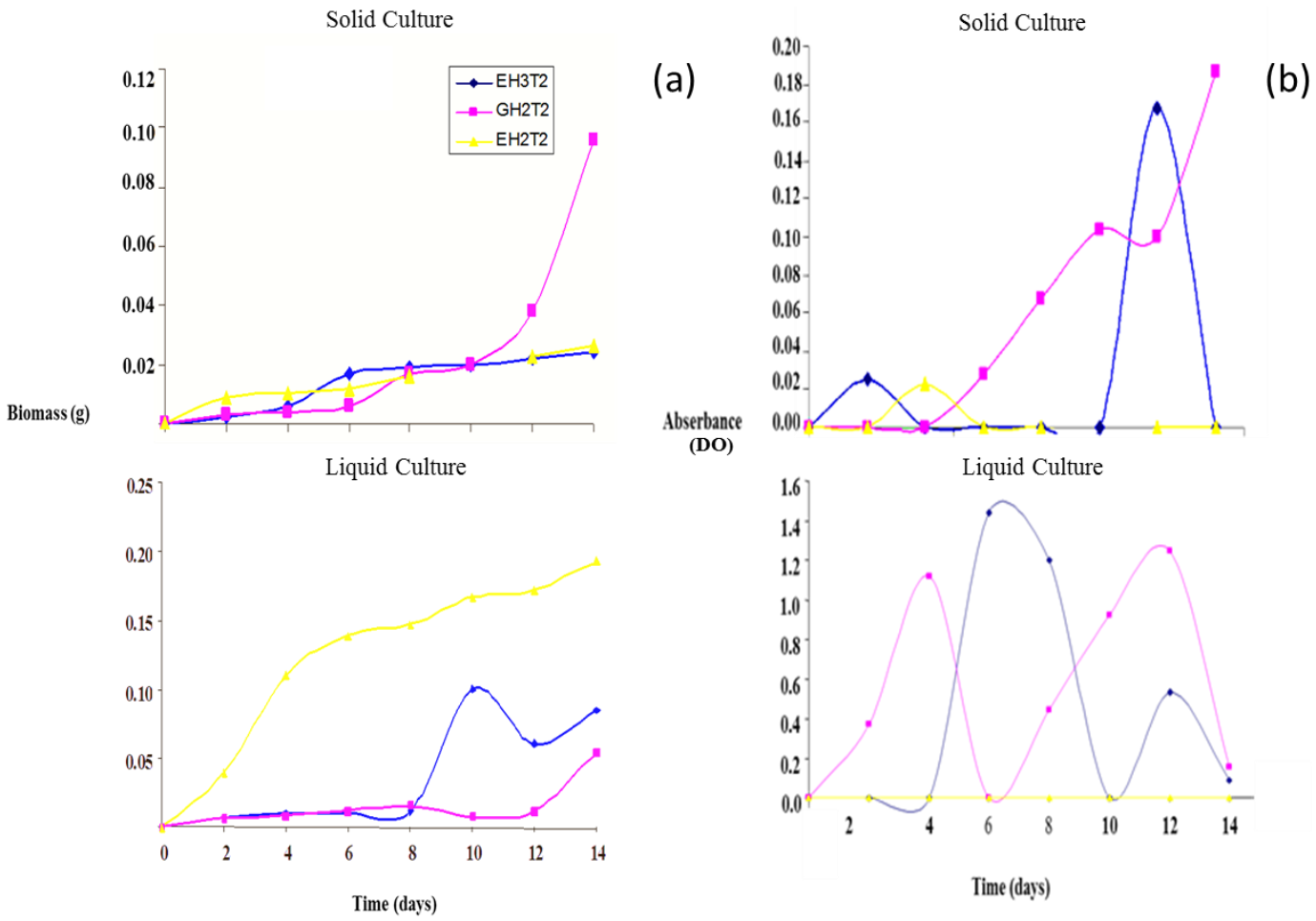

Figure 3. (a) Biomass (g) production. **(b)** Pigment (DO) production read at 414 nm by three *Penicillium* strains (GH2, EH2 and EH3) during submerged and solid state fermentation.

culture. EH3 strain produced the greatest amount of pigment on SSF, making a difference respect to EH2 strain, which produced very low levels of pigment (less than 0.001 U abs) under both fermentation systems. In general, *P. purpurogenum* GH2 strain grew and produced pigments in culture media with different carbon source (xylose and mannitol) in the absence or presence of micronutrients, with a maximum level of pigment production of 0.029gL/h, using xylose as substrate at 15 g/L (Hernández-Rivera et al., 2008).

The GH2 and EH3 strains were able to grow and produce pigments on SSF. On the other hand, the EH2 strain was able to grow, but it did not produced pigments on SSF. One explanation to this may be that this strain needs different conditions to grow that those needed to produce pigments. This difference in pigment production may be associated with the EH2 primary metabolism, making a difference in respect to the EH3 strain. Although EH2 and EH3 are taxonomically and molecularly very similar, they showed differences in their metabolic activity or gene expression of secondary metabolites, suggesting the existence of subspecies. The use of metabolic tools (selection and screening of specific metabolites such as mycotoxins) to identify differences among species or subgenera in filamentous fungi such as *Penicillium* spp., especially in microorganisms with potential use in pigments production for food industry has been suggested (Mapari et al., 2005). Hence, it is necessary to perform an appropriate metabolic analysis of the tested strains in order to demonstrate their potential use as pigment-producers. The results obtained in this study may help to perform a pre-selection among the pigment producer strains.

Random amplification of polymorphic DNA (RAPD)

RAPD amplification from the three fungal pigment-producing strains produced amplified fragments with molecular weight between 300 and 700 base pairs (bp) obtained with the A08 primer. GH2 and EH2 strains showed a common band of 550 bp being different in the 300 and 450 bp bands in GH2 strains and in the bands 350, 650 and 700 bp in the EH2 strain. Amplification with the D06 primer were useful to differentiate between EH2 and EH3

Table 2. Segments amplified by RAPD from three *Penicillium* pigment-producer strains.

Fungi key	Primer		
	A02	A08 (bp)	D06 (bp)
GH_2	-	550, 450 and 300	700
EH_2	-	700, 650, 550 and 350	350
EH_3	-	-	350 and 1500

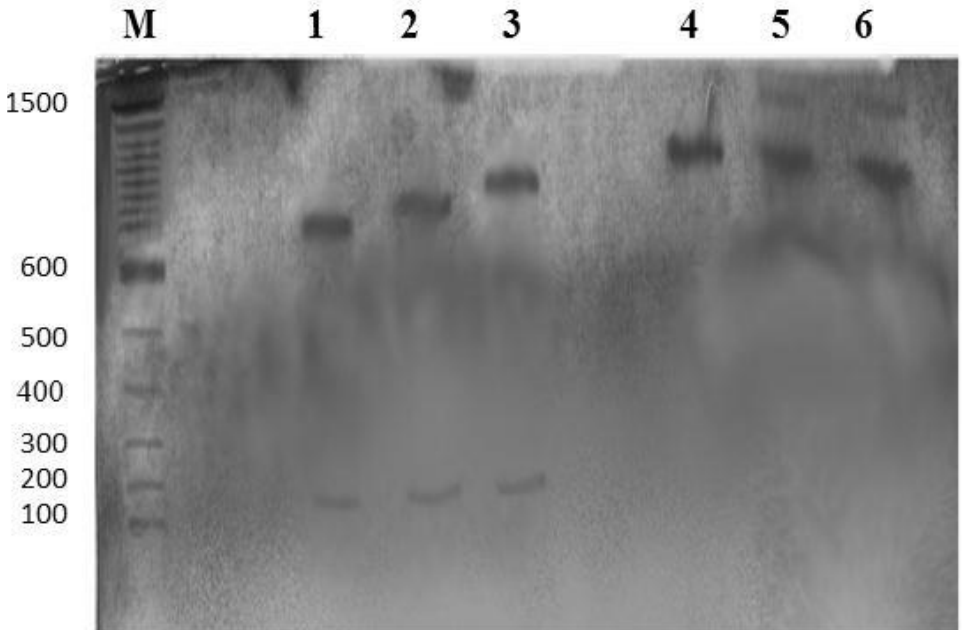

Figure 4. Restriction patterns generated from digestion of the fungal IGS and ITS regions with the *Hind* III enzyme. Lane M, Invitrogen 100 bp ladder molecular marker; lane 1, IGS region from GH2 strain; lane 2, IGS region from EH2 strain; lane 3, IGS region from EH3 strain; lane 4, ITS region from GH2 region; lane 5, ITS region from EH2 strain; lane 6, ITS region from EH3 strain.

strains, which have morphological similarities, because the EH2 strain did not showed the 1500pb band found in EH3 strain (Table 2). The use of RAPD amplification allows differentiating the molecular level among the three pigment-producing strains. Using the D06 and A08 primers, it is possible to differentiate various *Penicillium* species (Dupont et al., 2005). However, it was not possi-ble to obtain RAPD fragments using the A02 primer from any of the three *Penicillium* strains; the use of this primer for RAPDs from *Penicillium* strain is not recommended.

Internal transcribed (ITS) and intergenic (IGS) spaces amplification

The IGS amplified fragments from the three pigment-producing fungal strains and digested with the Hind III and Xho I restriction enzymes allowed the confirmation that GH2 strain is different from EH2 and EH3 strains. GH2 strain differ from EH2 and EH3 strains and these differ between them according to the IGS amplified regions (Figure 4), indicating that they may belong to different fungal species (correlating with morphological characterization). However, the relationship found among the EH2 and EH3 strains do not necessarily indicate that they belong to the same strain, but this relationship gives a clue of the narrow genetic relativeness between both strains (Figures 4 and 5). The *Penicillium* genus has at least 150 species, which have very similar morphology (Pitt and Hocking, 1997).

On the other hand, the amplified and digested ITS fragments did not give enough polymorphism to discriminate among the three pigment-producing fungal strains. The taxonomic information based on ITS region often gives enough information for specie identification (Pianzzola et al., 2004), and to solve classification problems to intra-generic level for many fungi (Le Cam et al., 2001). It is mentioned that it is not enough to use these sequences as the sole criterion for identification when low polymorphism is detected (Pianzzola et al., 2004). This is corroborated by the results obtained in this study where the

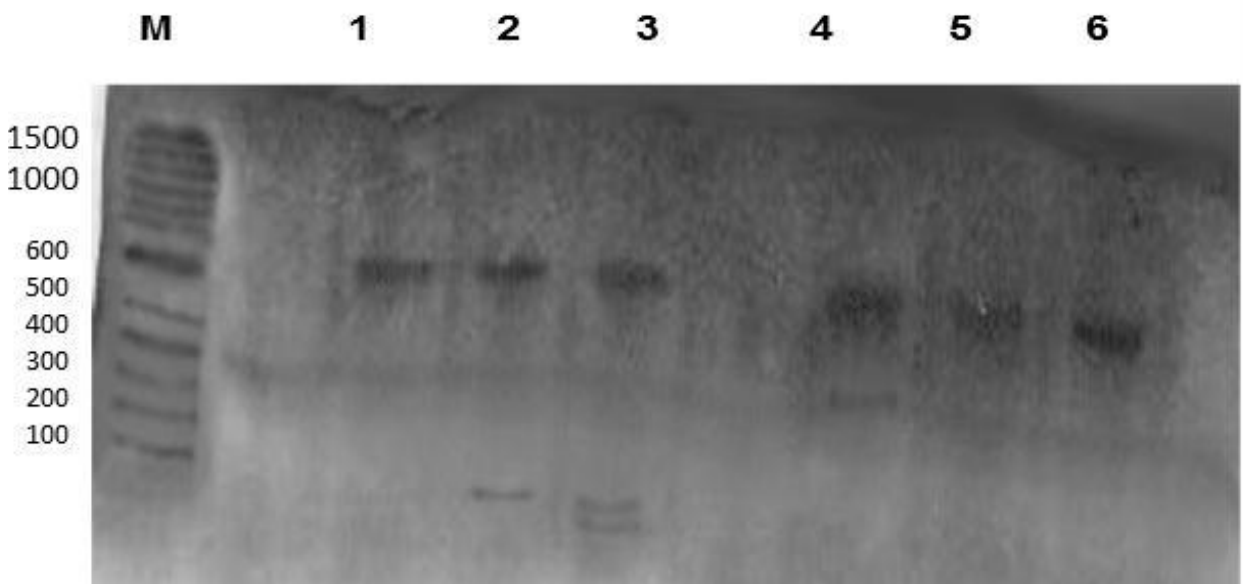

Figure 5. Restriction patterns generated from digestion of the fungal IGS and ITS regions with the *Xho 1* enzyme. Lane M: Invitrogen 100 bp ladder molecular marker, lane 1, IGS region from GH2 strain; lane 2, IGS region from EH2 strain; lane 3, IGS region from EH3 strain; lane 4, ITS region from GH2 region; lane 5, ITS region from EH2 strain; and lane 6, ITS region from EH3 strain.

restriction patterns generated on the ITS amplified fragments with some restriction enzymes (Hind III) showed little or no differences (Figure 4), and the generated ITS polymorphism was very low; for this reason, it was necessary to perform the analysis of the IGS region.

Conclusions

According to their morphology, it was possible to identify the three pigment-producing strains isolated from the Coahuila semi-desert, Mexico as *P. purpurogenum* (GH2) and *P. pinophilum* (EH2 and EH3). It was found that at 24°C and pH 10, the three strains produced more pigment in compared to other tested conditions. This is to our knowledge the first morphological, physiological and molecular characterization of pigment-producing *Penicillium* strains isolated from the Mexican semidesert.

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