Molecular identification of shiitake [Lentinula edodes Berk (Pegler)] and production of secondary metabolites with biotechnological potential

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Abstract: The shiitake mushroom (Lentinula edodes) is the second most-consumed mushroom in the world; in Colombia, it is cultivated and commercialized on a small scale in some supermarkets. Little is known about the occurrence, nutritional and medicinal properties of Shiitake produced in Colombia. In this study, four shiitake isolates grown in Colombia (LEUCO1, LEUCO2, LEUCO3, and LEUCO4) were sequenced in their ITS genes and evaluated for the production of three medicinal metabolites, eritadenine, ergotroine and β-glucans (1,3-1, 6), using submerged culture. Genetic analysis revealed that all the isolates were close and related to the Japanese strain Cr62. LEUCO1 and LEUCO2 showed a distance of 0.000, as well as LEUCO3 and LEUCO4. All four isolates produced eritadenine in a range of 26.3 - 8.6 mg / L, with the best performance of LEUCO1 at 26.3 mg / L (p <0.05). Ergotroine was produced with similar statistical yields in all the isolates with an average of 0.7 mg / g of dry weight biomass (DW). β-glucans (1,3-1,6) were produced with yields of 5.6 - 3.8% of DW biomass, with the best values for LEUCO2 and the lowest for LEUCO4 (p <0.05). In conclusion, we identified low genetic diversity in the four isolates, corresponding to two haplotypes with minimal genetic difference between them, related to the Japanese strain Cr62, indicating that Colombian farmers cultivate almost the same strains of shiitake. Secondary metabolites, eritadenine, β-glucans and ergotroine were found in promising yields useful for the pharmaceutical and food industries. More studies should be conducted to improve the yield of shiitake metabolites through new growing conditions for industrial production and to find metabolic pathways and related genes.

Key words: Eritadenine, ergotroine, β-Glucans, ITS genes, Shiitake mushroom.

Introduction

Gourmet shiitake mushroom has been consumed for more than a thousand years in eastern Asiatic countries and has been studied due to the essential medicinal properties1. The mushroom is mainly cultivated in China, Korea, and Japan, and currently is the second more consumed mushroom in the world, after the mushroom (Agaricus spp) with 25% of total mushroom world production2. Most shiitake research has been focused on the medicinal properties, nutritional qualities, and contents of important bioactive substances beneficial to human health. Some known activities are antitumor, immunostimulatory, antibacterial, hypcholesterolemic, and antioxidant3. Some of these shiitake substances are proposed to use in pharmaceutical and food industries4.

In Colombia, shiitake cultivation initiated in the 80’s decade; since then, the mushroom is increasingly becoming more popular in country5. Shiitake is produced in the Andes under tropical weather conditions that are different from the regions where the mushroom is originated. In recent years, the consumption of shiitake has increased. The mushroom is sold in supermarkets as a fresh product and is used for food and medicine. However, little is known about the genetic origin of Colombian Shiitake, genetic diversity, and parent strains, as well as the contents of bioactive compounds of shiitake cultivated under the tropical conditions of Colombia. Since the genetic instability has been frequently observed in edible mushrooms6, environmental conditions might generate genetic and/or biochemical changes and differences in the secondary metabolite profile7. These could influence the potential of Colombian shiitake usability in biotechnological processes, considering that the mushroom potential to produce secondary metabolites has been already demonstrated8.

Different studies have been carried out to establish the genetic characterization of shiitake mushrooms in different countries. Xiao et al.9 sequenced the whole genome and made a comparison between cultivated and wild strains from China, to evaluate genetic diversity, and George et al.10 used ITS sequences in shiitake to validate identifications of L. edodes strains based on morphology characters. ITS sequencing is very accepted in scientific literature to determine identify, genetic diversity, intraspecific variation with high confidence as a universal barcode marker for fungi kingdom, and prokaryotes in general11. In the case of shiitake mushroom, molecular identification is essential because there are some other Lentinula species with similar morphological features, as the edible and tropical American native Lentinula boryana12. Besides, genetic characteristics are determinant in the production of secondary metabolites13.

Obtention of secondary metabolites of shiitake under submerged culture has become a critical biotechnological process for pharmaceutical and food uses14. The purine alkaloid eritadenine [2(R), 3(R)-dihydroxy-4-(9-adenyl] butyric acid), is the primary, secondary metabolite responsible for the hypcholesterolemic action of shiitake, and has been proposed as pharmaceutical ingredient. HPLC quantitation of eritadenine in carpophores of some shiitake strains has been made by Ennan et al15 and Shulei et al16. Additionally, the production of eritadenine by submerged cultivation of shiitake mycelium have been described in different studies with unsatisfactory yields17,18. Recently, new culture techniques have improved the obtention of this metabolite19.

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The secondary metabolite ergothioneine (2-mercaptohistidine trimethylbetaine = ERG) is one of the main antioxidant substances in shiitake, with potent antioxidant properties for cells and DNA, and is anti-inflammatory. Different authors evaluated ergothioneine contents in many mushrooms. Additionally, other authors showed interest in establishing adequate cultivation parameters, to enhance the ergothioneine production, in shiitake mycelium and fruitbodies.

β-Glucans (1,3-1,6) are anticancer substances conformed by glucose polymer chains, bonded in positions 1-3, and short-ly ramified every 3 glucose bonded at 1,6 positions; they contribute significantly to the structure of cell walls in shiitake. Contents of β-glucanes (1.3 – 1.6) have been reported by Nitschke et al. in mycelium and carpophores of shiitake, using a simple spectrophotometric Protocol quantitation with Congo red. Also, Bak et al. quantitated α and β glucans in ten shiitake cultivars in mycelium and fruitbodies, although, the authors do not mention the conditions used to culture the mycelium. Nevertheless, in literature, it was not possible to find comparisons of β-glucanes (1.3 – 1.6) yields between different strains of shiitake, even more under submerged cultivation.

The comparison of yields of biotechnological, ergothioneine and β-glucanes (1.3 – 1.6) in various shiitake isolates, could be useful to select strains in biotechnological production of the metabolites. In this study were identified shiitake isolates cultivated in Colombia by sequencing ITS genes and evaluated the production for the secondary metabolites eritadenine, ergothioneine and β-Glucans (1.3-1.6) under submerged culture.

Methods

This experiment was carried out at the laboratory of biotechnology, Universidad Católica de Oriente (Rionegro-Colombia), and institute Centro de la Ciencia e Investigación Farmacéutica (CECIF).

Shiitake cultures propagation

Carpophores of shiitake were obtained at different local markets, from four different producers. These were used to obtain axenic cultures by extracting internal tissue with totipotent cells. Mycelium was propagated on malt extract agar (MEA) in Petri dishes and incubated at 24 °C by twelve days. The isolates obtained were named LEUCO1, LEUCO2, LEUCO3 and LEUCO4, and used for genetic characterization and obtention of secondary metabolites.

Genetic characterization of isolates

Identification of the isolates was carried out according to Sha et al. DNA was extracted from every isolate using the Invitrogen Purelink Genomic DNA kit. DNA extracted was quantified by light absorption at 260nm (Nanodrop) and visualized in agarose gel. PCR was performed using the primers 5TCCTTAGGTGAAACCTCGGG3 and 5TCCTTGGCTTAATTGATATGCT3 for ITS1 e ITS4 respectively. Both primers amplify fragments containing around 600 bp. Products of PCR were purified with BSA using the QIAquick PCR purification kit. Fragments containing around 600 bp. Samples were sequenced by the sanger/capillary method, with a MeCN gradient, starting with 2% during the first 5 min, changed to 80% from 5.1 to 10 min, then finally set again to 2% from 10 to 20 min. Values obtained were compared against a calibration curve constructed with the authentic standard substance (Santa Cruz Biotechnology Inc).

β-Glucans (1.3-1.6) quantitation

β-Glucans were analyzed in all the isolates by three successive extractions in the same sample, according to the procedure of Nitschke et al. Extractions were as follows: 1) KOH fraction was obtained by extracting the pulverized-dried mycelium with 1M KOH, in a proportion of 40:1 (w/v) with stirring, during 20 min at 60°C. The extract was cooled down, filtered, neutralized with 6M HCl to pH=7.0, and adjusted to 100 ml with distilled water; 2) HCl fraction was obtained using the filtered cake from the last extract, suspended in 0.55 M HCl, and heated at 100°C for 1h. The extract was filtered and the filtrate neutralized with 6M NaOH to pH=7.0 and adjusted to 100 ml with distilled water; 3) NaOH fraction, was obtained from filter cake, extracted in 1M NaOH at 60°C for 20 min, then was cooled down, filtered, neutralized with 6M HCl to pH=7.0, and adjusted to 100 ml with dH2O.

The quantification of β-Glucans (1.3 –1.6) was performed using the spectrophotometric method with Congo red, developed by Nitschke et al. Into the spectrophotometric (spectroquant Pharo 400, Merck, Germany) cuvette, were put 700 µL of fungal extract, 700 µL of Citric acid Buffer (0,2M Citric acid/NaOH, pH=7, Merck, Germany), and 100 µL of Congo red. Also, Bak et al. quantitated α and β glucans in ten shiitake cultivars in mycelium and fruitbodies, although, the authors do not mention the conditions used to culture the mycelium. Nevertheless, in literature, it was not possible to find comparisons of β-glucanes (1.3 – 1.6) yields between different strains of shiitake, even more under submerged cultivation.

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red solution (0.08 g in the citric acid buffer, Merck Germany). Absorbance was read at 553 nm, and the $\beta$-Glucans (1,3-1,6) concentration was determined using a calibration curve constructed with a series of solutions of standard schizophyllan (InvivoGen, USA). $\beta$-Glucans (1,3-1,6) found in the three extracts were added and expressed as total $\beta$-Glucans (1,3-1,6) in % of the dry weight of mycelium.

Statistics
Every metabolite was studied by random experimental design with four treatments (isolates) by triplicate, then data were analyzed by ANOVA (Tukey test), or Kruskal-Wallis (Dunn posthoc test) with R studio 4.0.2 of 2020.

Results and discussion

Genetic characterization
We could establish the identity of four shiitake mushrooms of unknown origin cultivated in Colombia by sequencing two ITS genes. PCR products from the isolates were purified, visualized with electrophoresis and sequenced for ITS1 and ITS4 genes (Figure 1a). The sequences obtained were 649 bp long in all four isolates, identified as Lentinula edodes species, and were compared with the elite strain Cr62 from genebank (https://www.ncbi.nlm.nih.gov/genbank/). Genetic distance values were generated along with a Neighbor-Joining phylogenetic tree (Figure 1, B and C).

ITS sequences revealed low differences between isolates (matrix fig. 1 B). The sequences were gathered in two homolog groups (haplotypes) 1) LEUCO1-LEUCO2 and 2) LEUCO3-LEUCO4, with 0.000 distance in between, close to the elite strain Cr62. Distances between Cr62 and the isolates were equally low. The first group had a difference of 0.15% with the strain Cr62, with one base substitution and one deletion. The second had a difference of 1.57%, nine bases substitution in a different order, and two deletions. Contraditorily, Sha et al. found a variation of up to 138 bases in wild samples of the gourmet mushroom Thelephora ganbajun from China. These resulting distances confirm a low genetic diversity in the evaluated isolates. Additionally, Nilsson et al. found from an exhaustive reviewing of sequences covering the fungi kingdom, an average intraspecific variability of 2.51% based on ITS sequences. Accordingly, Xiao et al. found a reduced genetic diversity in Chinese shiitake cultivars compared to wild strains, probably caused by human selection and breeding.

Eritadenine assay
All four isolates produced eritadenine (8.6 - 26.3 mg/L). Best yields were attained by LEUCO1 (26.3 mg/L), with statistical differences with the rest of isolates (Table 1). Here, yields were higher than those published by other authors with maximal yields of 8.6 and 25 mg/L, using broths of complex nutritional composition, and lower than 88 mg/L obtained in other experiments with alginate immobilization.

Few reports exist in the literature related to the obtention of eritadenine by different shiitake strains in submerged culture. Enman et al., found in carphophores of four shiitake strains maximal yield of 6.33 mg/g, with no significant statistical differences, also Shulei et al. reported 1.99 mg/g in carphophores, without mentioning the strain. Both authors did not report the

Table 1. Yields of metabolites on average after 20 days of submerged fermentation with shiitake mycelium.

| Isolate | Eritadenine mg/L | Total $\beta$-Glucans 1,3-1,6 DW % | Ergothioneine mg/g DW | pHf | Biomass g/L |
|---------|------------------|----------------------------------|----------------------|-----|------------|
| LEUCO1  | 26.3 ± 1.4 a     | 4.78 ± 0.4 ab                    | 0.73 ± 0.04 a        | 3.28 ± 0.03 a | 1.21 ± 0.04 a |
| LEUCO2  | 13.2 ± 1.3 b     | 5.61 ± 0.8 a                     | 0.72 ± 0.08 a        | 3.40 ± 0.2 a  | 1.82 ± 0.06 ab|
| LEUCO3  | 13.3 ± 0.1 b     | 4.33 ± 0.4 bc                    | 0.62 ± 0.2 a         | 3.43 ± 0.2 a  | 1.92 ± 0.07 b |
| LEUCO4  | 8.6 ± 0.1 c      | 3.81 ± 0.2 c                     | 0.76 ± 0.06 a        | 3.52 ± 0.2 a  | 1.89 ± 0.07 b |

Figure 1. Genetic characterization to the isolates. A: purified products of PCR; B: Distances matrix between the isolates, and C: Phylogenetic tree for all four isolates together with Cr62 elite shiitake strain.
methods used to produce the carpophores, then possibly, the differences were not only due to genetic differences but also to cultivation conditions, as samples were obtained from different producers. The latter probably used different environmental and nutritional conditions.

Here we have three groups of shiitake isolates (LEUCO1, LEUCO4, and LEUCO2-LEUCO3) that express eritadenine genes at different levels. Seemingly those differences in eritadene expression do not have a clear relationship with the distances obtained (Fig 1), which could be a logical assumption as ITS genes do not encode for enzymes, then do not participate in eritadene biosynthesis pathway. Accordingly, Schwessinger et al. found gen expression variation of 6% in Puccinia striiformis, which occur in dikaryotic isolates (diploids) with sexual reproduction, as the shiitake isolates evaluated in the present research. Also, the production of purine precursors to eritadine (AICAR and SAICAR) in fungal cell wall30,31. And the β-glucan synthase, the primary enzyme responsible for fungal cell wall construction30. Those β-glucans (1,3 – 1,6) the yields slightly more than twice β-glucans (1,3 – 1,6) the yields reported by Nitschke et al.24. This author obtained 2.58 g/100 in shiitake mycelium, using the same extraction and quantitation protocols than this experiment. Nonetheless, the strain name was not reported, and the cultivation conditions were broth of yeast and malt extracts, at 80 rpm (different than used in this experiment).

Likewise, other studies reported differences in β-glucans contents in different cultivars of shiitake mushroom4 and in Agaricus subrufescens38. Those differences in β-glucans (1.3 – 1.6) were probably due to the homeostasis between β-(1,3)-glucan synthase, the primary enzyme responsible for fungal cell wall construction30. And the β-glucans degradative counterparts β-(1,3)-glucanasases31. In shiitake, various glucanasases coding genes have been identified, which are upregulated in postharvest shiitake fruiting, leading to the autoysis of cell wall32,33.

In Lentinula edodes composition of the cell wall is well known, mainly consisting of polysaccharides like chitin and β-glucan3. Those are synthesized by the enzyme β-(1,3)-glucan synthases that have a catalytic subunit FKS, plus a regulatory one RHO, both encoded by FKS and RHO genes34,35. However, there is inadequate knowledge about the transcriptional regulation of this enzymatic biosynthesis35. We hypothesize that nutrition and environmental conditions used in this experiment, especially the glucose concentration of the broth, produced a positive balance in favor of β-(1,3)-glucan synthase activity in LEUCO2 (the more productive isolate), but not for LEUCO4.

Ergothioneine assay

All isolates produced similar ergothioneine yields, ranging from 0.62 to 0.75 mg/g DW of mycelium, with an average of 0.7 mg/g, to all the isolates. These concentrations did not show significant statistical differences between isolates but were higher than the concentrations reported by Tepwong et al.22, who found 0.5 mg/g for Shitake mycelium pellets in submerged culture. And seventeen times higher than the yields reported by Jang et al.23 who used shiitake submerged cultures supplemented with various nitrogen sources. Unexpectedly, the isolates used in this experiment contained eight times more ergothioneine than the fruiting body of the popular Ganoderma lucidum25.

Few reports in literature compare ergothioneine content in different strains of edible mushrooms related to Shiitake. Contrary to this research, Chen et al.20 found different ergothioneine concentrations in the mycelium of three strains of Cordyceps militaris, and in three commercial carpophores of Pleurotus ostreatus from China, Korea, and Japan. However, the authors do not report cultivation methods to obtain carpophores or mycelium; then, it is possible to speculate that differences were related to the possible different cultivation conditions. Hence, to obtain accurate comparisons, fungal cultures for chemotaxonomic analysis should be grown in the same medium and incubation conditions, to ensure that differences correspond to fungal genetic diversity and not to environmental conditions25.

Nitrogen source is critical for ergothioneine biosynthesis, amino acids like cysteine, methionine, and histidine are precursors of the substance36. By adding methionine to liquid broths, ergothioneine yields are increased up to twice, compared to fermentation without the addition of aminoacids37. This is due to histidine is a precursor of ergothioneine23,27. Then the low ergothioneine yields obtained in this experiment were probably due to the lack of precursors amino acids in the yeast extract used. For instance, Lin et al.38 found in submerged cultures of Pleurotus citrinopileatus a higher ergothioneine yield 10.65mg/g, by using tryptone instead of yeast extract. Probably tryptone contains 1.6% of methionine, while yeast extracts only 0.8%. In this sense, we hypothesized that the use of nitrogen sources rich in the amino acids mentioned above might improve ergothioneine yields produced in the shiitake isolates of this experiment.

Biomass production and final pH obtained in fermentations

All four isolates produced biomass after twenty days of fermentation in a range from 1.21 to 1.92 g/L. LEUCO2, LEUCO3, and LEUCO4 produced the higher biomass (1.82, 1.92, and 1.89 g/L respectively) without significant statistical differences between them. At the same time, LEUCO1 produced the lowest value (1.21g/L), statistically similar to LEUCO2. Accordingly, shiitake strains show different utilization of nutritional components in liquid broths, which affects biomass yields. This was demonstrated by López-Peña et al.29, who found two high biomass producing strains 3.73 g/L and 8.79 g/L, from six evaluated. In this sense, the differences between the isolates could be attributed to incubation temperature (24°C). Probably this temperature was not optimal for the isolate LEUCO1 and LEUCO2. According to Lee et al.39, every strain has an optimum growth temperature. For instance, Quaicoe et al.41 found different radial growth in three shiitake strains at 20°C, cultured in potato dextrose agar (PDA).

On the other hand, final pH had statistically similar values in fermentations of all isolates, ranging from 3.28 to 3.52. Different authors found similar pH at the end of fermentations. Enman et al.42, obtained a final pH of 3.5-3.6 for shiitake under submerged culture, with eritadenine yields of 25 mg/L. The same authors established that optimal pH for eritadene production could be in a range from 3 to 4. Likewise, Quaicoe et
Conclusions

In this investigation, we identified low genetic diversity in the four shiitake isolates examined, which corresponds to the most commonly produced cultivars in Colombia. These findings allow us to conclude that the four isolates formed two haplotypes LEUCO1-LEUCO2 and LEUCO3-LEUCO4 with little genetic difference between them, but related to the elite Japanese strain Cr62. All strains produced the pharmaceutical metabolites eritadenine in a promising concentration for industrial metabolites eritadenine in a promising concentration for industrial use. β-glucans (1-3, 1-6) and ergothioneine. LEUCO1 was more suitable for the production of eritadenine, while LEUCO1, 2, and 3 were more suitable for producing β-glucans. Furthermore, all four isolates produced the same amounts of ergothioneine. Finally, biomass was better produced by LEUCO2, 3 and 4. Further research could be aimed at improving shiitake metabolite yields and optimizing culture and incubation systems, for industrial production, and finding metabolic pathways and genes related.

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