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Presence of Enzymes and Secondary Metabolites Clusters in DNA Sequence of *Aspergillus salvadorensis* in the Production of Natural Black Pigments

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ABSTRACT **Original research paper**

Objective. To determine by the Next-Generation Sequencing Illumina method to identify the genes related to the pigmentation of the fungus in the DNA sequencing of enzymes and secondary metabolites. As well as in determining the phenotypic and genotypic characteristics in general of the species *A. salvadorensis*. Methodology**.** For the identification of cluster genes in enzymes, proteins, and secondary metabolites, MACROGEN For the identification of cluster genes in enzymes, proteins, and secondary metabolites, MACROGEN used the following systems: the EggNOG system summary: Orthology Frequency within COG (Clusters of Orthologous Groups) Categories in sequencing reading., MetaCyc is a comprehensive database that provides in-depth information on metabolic pathways, biochemical reactions, enzymes, and associated compounds. UniRef (UniProt Reference Clusters) organizes groups of protein sequences from the UniProt Knowledgebase including isoforms and selected UniParc entries into clustered sets to enhance sequence analysis and annotation efficiency. EggNOG: Relative Abundance in Hierarchical Categories of COG (Clusters of Orthologous Groups) using CPM, KEGG Orthology (KO) and KEGG summary: Orthology Frequency within Main and Sub-Categories. DNA-seq. Conclusions. 14 enzymes and secondary metabolites were found in the production of black pigments produced by the fungus by oxidative stress.

Keywords: *Aspergillus salvadorensis*, Macrogen, Metabolites, Enzymes.

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[1]Introduction

This organism exhibits a unique characteristic: it requires the presence of the fungus *Aspergillus salvadorensis* to interact with the tannins found in the seed, producing its distinctive black coloration. Alternatively, the fungus is capable of generating the dye independently. The seed originates from the *nacascol* tree, scientifically known as *Caesalpinia coriaria*, which belongs to the family Caesalpiniaceae, genus *Caesalpinia*, and species *coriaria*. This species is traditionally used in the dyeing of animal hides.

Caesalpinia coriaria is a leguminous plant characterized by a stem ranging from 3 to 11 meters in height. Its leaves are composed of paired pinnae measuring 5 to 10 cm in length, each bearing more than ten leaflets approximately 4 to 8 mm long and 2 mm wide, with rounded tips. The seeds are dark brown to black in color and serve as a natural habitat for *Aspergillus salvadorensis*. The spores of this fungus are distinctively arranged in separate spicules, are pigmented, small in diameter, and appear black.

When cultured in a specialized medium, the fungus produces a black pigment within 24 hours, particularly in the posterior region of the growth tube. This pigmentation is

[1]***Fungal Name FN 573057: Aspergillus salvadorensis Antonio Vásquez Hidalgo (2025-10-17); GenBank ID: PRJNA1306032.***

enhanced when oxidizing agents are added to the flavoured agar medium, either through interaction with iron or through the fungus's own secondary metabolite production. Phytochemical screening of *Caesalpinia coriaria* has identified the presence of tannins, triterpenes, glycosides, and flavonoids.

Justification

A comprehensive study was undertaken to extract and identify DNA from various species within the genus *Aspergillus*, aiming to determine which species are native to the region. The research initiative began in 2006 with phenotypic analyses conducted in the Microbiology Department of the Faculty of Medicine at the University of El Salvador. In 2008, further morphological studies were performed using scanning electron microscopy at CENSALUD. Preliminary molecular results, including PCR analyses, were obtained in Mexico. In 2024, advanced sequencing using the Illumina method was conducted at MACROGEN INC laboratories in South Korea. This allowed for the complete extraction, quantification, and sequencing of genomic DNA (gDNA, qDNA, cDNA, dsDNA, seqDNA), enabling precise molecular identification of native *Aspergillus* species circulating in northern regions of the country. It is clarified that sequencing laboratories provide raw data in general in millions of sequences, but they must be aligned to determine the species since according to the phylogeny it can be the product of a mutation, deletion in positive conserved sites, moderate or weak alignment, gaps, using programs such as GenBank, Clustal, Mega12, PHYTON and others.

Objective: To determine by the Next-Generation Sequencing method to identify the genes related to the pigmentation of the fungus in the DNA sequencing of enzymes and secondary metabolites. As well as in determining the phenotypic and genotypic characteristics in general of the species.

Methodological design

Research Methodology Overview

The study was conducted in four distinct phases:

Phase 1: Seed Collection and Initial Fungal Inoculation

Seeds of *Caesalpinia coriaria* (commonly known as nacascol) were gathered from five different sources in the northern region. After collection, the seeds underwent a selection process based on color and appearance to ensure quality. Fungal inoculation was carried out using Sabouraud Dextrose Agar (SDA) in both petri dishes and test tubes. Cultures were incubated, and fungal growth was monitored under 40x and 100x magnifications using a basic light microscope over several weeks, with observations compared to a reference chart for identification.

Phase 2: Tannin Extraction and Microculture Preparation

This stage involved extracting tannins from the seeds and cultivating the fungus. Spore suspensions were created by isolating conidia from existing cultures under a microscope, then reseeded into SDA media in both plates and tubes. The fungus was also inoculated in 250 mL Erlenmeyer flasks containing 50 mL of SDA and incubated at 28°C for 48 hours. Afterwards, 100 mL of room-temperature distilled water was added and stirred with a magnetic stirrer for five minutes to achieve a uniform spore suspension. Manual mixing was used when settling occurred. Spore concentrations were quantified using a Neubauer chamber.

A microculture was established using a sterile wet chamber setup, with inoculations performed on SDA blocks placed on microscope slides, which were incubated at 25°C for seven days. Post-incubation, the slide coverslips were treated with Lactophenol Blue, and fungal structures were examined microscopically under 100x magnification.The fungus was isolated directly from black nacascol seeds stored at room temperature in sealed bags. No seed cleaning was done before fungal isolation; instead, the surface was scraped, and the sample was placed on SDA for identification and purification.

Phase 3: Laboratory Cultivation and Morphological Characterization

The isolated fungal inoculum was cultivated in SDA tubes, incubated for one week, and repeatedly subcultured until pure strains were obtained. These isolates were then transferred to 150 mL of PW (peptone water and nutrient broth) liquid media and incubated at 37°C for 48 hours, followed by 20–25°C at room temperature. The resulting biomass was dried at 42°C for two days and then left at ambient temperature (25°C) for three weeks for subsequent analysis.Fungal morphology was analyzed through culturing on SDA and incubation at 37°C for 72 hours, followed by an additional seven days at room temperature. Microscopic examination was carried out using cotton blue-stained preparations at 10x, 40x, and 100x magnification. Observations of structural features were compared to online taxonomic databases for species identification.

Phase 4: Molecular Characterization

For genetic analysis, three fungal samples were prepared: one pure strain in a glass tube, one DNA sample, and one untreated fungal specimen. These were sent to MACROGEN INC, South Korea, for sequencing and analysis. The Sanger method was employed for DNA sequencing, involving three main steps: synthesizing new DNA fragments, separating them via electrophoresis, and identifying nucleotide sequences. Small DNA fragments (amplified or randomly fragmented) were sequenced to study the fungal genome. Illumina sequencing technology was used for high-throughput DNA analysis. This involved PCR bridge amplification for creating DNA clusters, followed by base

detection using fluorescent markers. Sequencing was conducted at both ends of DNA fragments, covering genomic DNA (gDNA), complementary DNA (cDNA), double-stranded DNA (dsDNA), and sequencing DNA (seqDNA). High-capacity computational tools were used to process and interpret the data.

gDNA Extraction and Analysis

Genomic DNA (gDNA) extraction focused on breaking down fungal cell walls, membranes, and nuclei using thermal, enzymatic, mechanical, or chemical methods. The goal was to release and purify DNA, eliminating protein and RNA contaminants without degrading nucleic acids. DNA quality was ensured by concentration and purity checks, and quantification was done using QuantiFluor® dsDNA System with a Victor Nivo microplate reader, which is more precise than UV spectrophotometry.

Aspergillus species, known for genomic variability, have genomes ranging from 27 to over 39 Mb and gene counts between 9,000 and 14,000. *A. sojae* holds the largest genome (39.5 Mb), while *A. niger* has the highest gene count (14,165).

Sequencing Cycle Quality and Bioinformatics Tools

Sequencing accuracy was assessed per cycle, with each cycle representing the incorporation of a single base (A, T, C, or G), detected by fluorescent signals. Cycle-by-cycle sequencing ensured high precision.

To identify genes associated with enzymes, proteins, and secondary metabolites, MACROGEN utilized:

i. **EggNOG**: Categorizes orthologous groups (COGs)
ii. **MetaCyc**: Details metabolic pathways, enzymes, compounds, and reactions
iii. **UniRef**: Clusters protein sequences from UniProt, including isoforms
iv. **KEGG**: Annotates gene functions across biological systems

These databases helped classify genes and pathways identified in *Aspergillus salvadorensis*, contributing to a deeper understanding of its biological potential.

Results

The morphological and genotypic characterization of the fungus is characterized in summary as follows: **Macroscopic characteristics**: colony on flavored agar is white, then changes to green, and then turns black, the reverse of the bevel is pigmented in color, height of the mycelium is low, appearance of the colony is dusty, moist black. **Microscopic characteristics**:

Morphological Description of Conidiophore and Conidia Structures

The conidial head is distinctly black, supported by a rough-textured conidiophore measuring between 1 to 4 mm in length. The conidiophore bears numerous internal conidia, ranging in size from 1 to 3 µm, with pigmentation varying from brown to black. Conidial heads are smooth with walls that range from rounded to irregular in shape and are arranged in uniseriate chains. The terminal tips are thin-walled, prominently smooth, and exhibit a brown to black coloration.A well-defined columella, or "gallbladder-like" structure, is present, from which a dense row of phialides arises. These phialides serve as the origin point for conidial development. The sporangium appears as a simple, globose peridial structure, and the sterigma is characteristically black.

Mature conidia are brown, spherical, and display surface ornamentation consisting of triangular-shaped spines or projections distributed across the entire surface. These are produced in abundance, while central spicules are less common but exceed ten in number. The conidia exhibit a stellate (star-like) spherical morphology with prominent spicules. From these spicules, filaments extend outward in linear chain formations, emerging directly from the protrusions.The entire fungal structure forms a dense, shell-like mass that does not absorb Lactophenol Cotton Blue stain, indicating resistance to staining or a dense wall composition. Immature asexual spores are initially colorless, large, spherical, and thin-walled. These immature spores are located near the base of the conidiophore (close to the foot cell) and gradually migrate toward the vesicle as they mature. Once fully developed, they are forcefully discharged outward through the phialides under internal pressure.

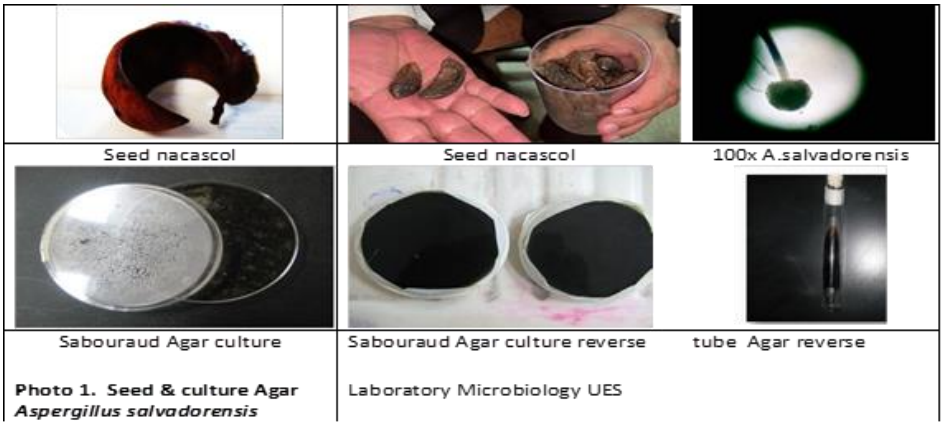

Photo 1. Slide of the Culture in plate and tube of *Aspergillus salvadorensis*.

In Fig 1. *Aspergillus salvadorensis* grown on Sabouraud Agar (SDA) is observed to turn black, which is strong evidence of melanin production. The slide describes the macroscopic characteristics of the A. *salvadorensis* colony as follows: Color of the colony in Sabouraud Agar: it is white, then changes to green, and then turns black. Reverse of the cologne: pigmented black. Appearance of the colony: "It is dusty and black in color." Images of petri dishes and tube culture also confirm this dark coloration. In mycology, this transition to a very dark black or brown color in the colony or reverse is the most important phenotypic indication of the production of melanin (a dark and protective pigment) or melanin-like compounds. Therefore, unlike many species of *Aspergillus*, they are yellow or light green in color. *A. salvadorensis* does produce a dark pigment (melanin) that accumulates in the conidia and mycelium as the colony matures. Extraction of the dye under experimental conditions.

The extraction of the black dye, usually melanin, produced by the Aspergillus fungus under laboratory conditions is a methodical procedure that begins with the cultivation of the microorganism. Since this pigment is inherently insoluble in many solvents, specific steps are required for its handling and treatment. The process begins with the Aspergillus Culture, where the fungus is sown in a Saboraud Agar medium, which provides essential nutrients such as sources of carbon (glucose) and nitrogen (peptones or salts). The crop is grown under controlled conditions, at a room temperature or slightly higher (approximately 25−30∘C) and with high humidity. This environment stimulates the production of large amounts of melanin, especially if the fungus is subjected to environmental stressors such as pH changes or UV radiation. Once the fungus has grown and synthesized the melanin in the medium, the Biomass is collected and prepared. The Aspergillus biomass, which contains the pigment, is separated from the liquid culture medium by methods such as centrifugation or filtration. This fungal biomass should be washed with distilled water to remove any residue from the culture medium that could interfere with extraction. Optionally, the biomass can be dried at a low temperature (40-50∘C) in an oven to facilitate the extraction stage. The extraction of melanin is the crucial step due to the insolubility of the pigment. The most common method is based on the solubility of melanin in alkaline solutions. The biomass is mixed with a 1-2% sodium hydroxide (NaOH) solution and gently heated (to about 60-80∘C) for several hours. This allows the melanin to dissolve in the alkali. The resulting solution is filtered to separate the insoluble biomass, and the filtrate, which contains the dissolved pigment, is neutralized with an acid such as hydrochloric acid (HCl) until a pH close to 7 is reached. This acidification causes the precipitation of melanin, which is later recovered by filtration and dried. Alternatively, other extraction methods may be employed. If the extraction alkaline is insufficient, organic solvents such as ethanol or methanol can be used; In this case, the biomass is mixed with the solvent, stirred with a slight heating, and the pigment is filtered out, allowing the solvent to evaporate to obtain the dried melanin. Concentrated hydrochloric acid is also sometimes used along with heating to dissolve and extract melanin, which is then precipitated and separated. After extraction, the melanin can undergo a Pigment Purification. Depending on the level of purity required for its end use, techniques such as additional precipitation, dialysis to remove impurities and other fungal metabolites, or column chromatography may be applied. Finally, the extracted and purified pigment goes through Drying and Storage. A dry melanin powder is obtained using a freeze dryer or a low-temperature oven (40-50∘C). This dry pigment should be stored in dark and dry conditions to prevent its breakdown or loss of color due to exposure to light or moisture. In summary, the melanin extraction protocol of Aspergillus involves the controlled cultivation of the fungus, the collection of the biomass and, predominantly, the extraction of the pigment through the use of alkaline solutions (NaOH), although other solvents such as ethanol or methanol can be employed. The process concludes with the purification, drying and storage of the dye, steps that can be adjusted to optimize extraction according to application needs. **Genotypic characteristics**: Genomic Sequencing Report (Shotgun Metagenomic Sequencing Report): This report consists of:

Raw Data Statistics

This section outlines the sequencing workflow and data processing as performed by **Macrogen** using the **Illumina next-generation sequencing (NGS)** platform.

A. Experimental Workflow – Macrogen (Illumina Platform)

The sequencing workflow follows the standard **NGS library preparation and sequencing pipeline**, although specific steps may vary depending on the library protocol used.

1. Sample Preparation

DNA or RNA is first extracted from biological samples. Only those samples that pass quality control assessments move forward to the library construction stage.

2. Adapter Ligation

The extracted nucleic acids are randomly fragmented, and sequencing adapters are attached to both the 5’ and 3’ ends. In some protocols, fragmentation and adapter ligation are performed in a single, streamlined step to improve efficiency.

3. Final Library Construction

Fragments with adapters are then amplified via polymerase chain reaction (PCR) using primers complementary to the adapter sequences. After amplification, the resulting library undergoes quantification and quality control to ensure it meets the required standards for sequencing.

4. Cluster Generation via Bridge Amplification

The prepared library is loaded onto a flow cell, where the adapter-ligated fragments bind to oligonucleotides on the surface. Each DNA fragment is then clonally amplified into clusters through a process known as bridge amplification. Once clustering is complete, the sequencing templates are ready.

5. Sequencing by Synthesis (SBS) Technology

Illumina's SBS method incorporates fluorescently labeled, reversible terminator nucleotides during the sequencing process. All four modified dNTPs are present in each cycle, minimizing incorporation bias and significantly lowering the error rate. This base-by-base synthesis approach enables highly accurate reading of sequences, including challenging regions such as repeats or homopolymers.

6. Fluorescent Detection Chemistry

Illumina platforms employ different channel chemistries depending on the sequencer model:

i. **Four-channel systems** use four distinct fluorescent dyes (one for each nucleotide).
ii. **Two-channel systems** use combinations of two dyes to distinguish all four bases.
iii. **One-channel systems** rely on a single dye with alternate detection mechanisms. The imaging data are processed using specialized software to accurately identify each incorporated base.

B. Raw Data Generation and Conversion

During sequencing, the instrument captures images of the fluorescent signals, which are analyzed in real-time by Illumina's Real-Time Analysis (RTA) software. This primary analysis step performs base calling and produces BCL (Base Call) or cBCL files in binary format.

The BCL files are then converted into FASTQ format using Illumina's bcl-convert software, which outputs readable sequencing data including base calls and quality scores.

Note: At this stage, adapter sequences are not removed from the reads. Further trimming and processing are typically performed during downstream bioinformatics analysis.

Identified sequencing metabolites

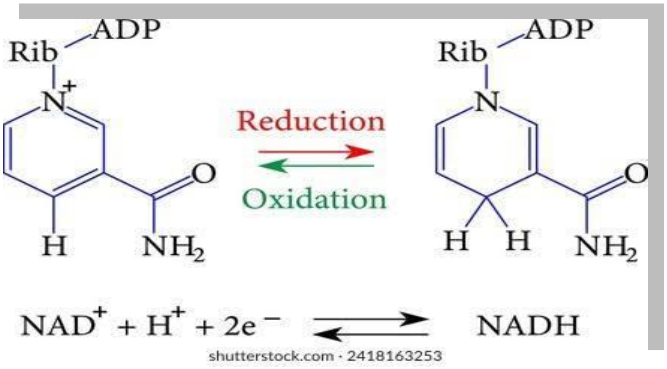

Fig 2. NADH molecule.

Source: Taken from Shutterstock with the ID 2418163253.free 2025

Fig. 2. Complex I, also called NADH dehydrogenase, is the largest enzyme complex in the respiratory chain. Its "L" shape consists of two main parts: a hydrophilic domain that enters the mitochondrial matrix and is responsible for receiving the electrons of NADH, and a transmembrane domain anchored in the mitochondrial inner membrane that contains about 60 helices. Inside this structure are several groups that facilitate the transport of electrons. The first is FMN (flavin mononucleotide), which accepts the electrons from NADH. Next, a series of iron-sulfur (Fe-S) centers, eight in mammals, transport these electrons to ubiquinone (coenzyme Q). This flow of electrons releases energy, which is used to pump protons from the matrix into the intermembrane space, thus creating an electrochemical gradient essential for ATP synthesis. Production of Pigments in Aspergillus Fungi. Several species of fungi in the genus Aspergillus produce pigments as secondary metabolites. [5] This process is usually activated in response to a lack of nutrients or environmental stress conditions. The synthesis of these pigments takes place mainly through the metabolic pathways of polyketide and psychimate. The production of pigments is closely linked to the formation of asexual spores (conidia), which are the structures that contain color. The genus *Aspergillus* generates a wide range of colors, including yellows, oranges, reds, browns, and blacks. A prominent example is melanins, dark pigments produced by species such as *A. niger and A. fumigatus*. These melanins serve a protective function, defending the fungus from damage caused by UV radiation and free radicals. [6]In addition, in pathogenic fungi, melanins help evade the host's immune system, increasing its ability to cause disease. Pigment production in *Aspergillus salvadorensis* and its relationship with metabolism. The fungus species *Aspergillus salvadorensis* is also a producer of pigments, as evidenced by the observation of black seed lots. Aspergillin, a type of pigment produced by this fungus, can manifest itself in black or blackish-green tones depending on various factors. [5,7] These factors include the specific species of Aspergillus, as well as environmental conditions such as nutrition, pH, temperature, and the presence of other compounds. In addition to dark pigments, several species of Aspergillus produce anthraquinones, which possess biological properties such as antimicrobial and antioxidant effects. Uses and indirect contribution of NADH dehydrogenase. Fungal pigments have a variety of applications in biotechnology. They are used as natural colorants in food, beverages, and other industries such as textiles and cosmetics. Additionally, many of these pigments have bioactive properties, such as antioxidant, antimicrobial and anticancer activity, which makes them of great interest to the pharmaceutical industry. Although NADH dehydrogenase does not produce pigments directly, its contribution is indirect but crucial. This enzyme, also known as complex I, has two main functions: Energy production (ATP): Its main function is to generate the energy needed for all metabolic processes of the fungus, including pigment synthesis. [5] Regulation of oxidative stress:

Aspergillus has alternative versions of this enzyme that help maintain redox balance and control the formation of reactive oxygen species, protecting the fungus from environmental stress. The production of melanin may be a complementary response to this protective mechanism. Genetic regulation of pigment production. The production of pigments in Aspergillus is a complex process regulated by the interaction of genetic and metabolic factors that respond to environmental conditions. The genes responsible for pigment production are usually clustered together and their expression is controlled by transcription factors. A key example of this is polyketide synthase (PKS) genes, which are involved in the synthesis of pigments such as **aspergillin** through specific metabolic pathways. Synthesis and Regulation of Pigments in *Aspergillus*. [8,9,10]Most of the pigments in the fungus *Aspergillus*, such as the melanin DHN (dihydroxynaphthalene), are polyketides. Its production begins with enzymes known as PKS (policétido sintasas), las cuales unen unidades de acetil-CoA y malonil-CoA. Factors that control pigment production. Pigment production is controlled by a complex interaction between genetic and metabolic factors: Genetic factors. DHN-melanin pathway genes: In some *Aspergillus* species, genes for DHN melanin production are clustered in the genome. These genes contain the instructions for enzymes that transform simple precursors into the final pigment. Regulatory genes: There are transcription factors, such as the brlA and wA genes in *Aspergillus nidulans*, that control the expression of pigment genes. For example, the wA gene in this species is responsible for changing the color of a pigment from yellow to green. Metabolic and Environmental Factors. Pigment production is a secondary metabolic process that is activated under specific conditions, mainly when the main nutrients are depleted. Substrate availability: The abundance of metabolic precursors (acetyl-CoA and malonyl-CoA) directly influences the amount of pigment produced. [11,12] For example, nacascol, being a rich source of carbohydrates, lipids and proteins, is an ideal substrate for *Aspergillus salvadorensis*. Environmental stress: Pigmentation, especially melanin, acts as a protection against various forms of stress, such as: Oxidative stress: Pigments function as antioxidants, neutralizing reactive oxygen species. UV radiation: Melanin absorbs UV radiation, protecting the spores' DNA from damage. Heavy metals: Some species of Aspergillus may modify their pigmentation in response to the presence of heavy metals. Cell Differentiation and Stress Regulation. Pigment production is closely related to conidiation (spore formation), a process of cell differentiation. The pigmentation of the spores gives them greater resistance to survive in harsh environments. The regulation of this process is a defense mechanism: environmental stress signals activate genetic transcription factors, which in turn kick-starts pigment production. Nutrient depletion triggers metabolic changes in fungi, redirecting their energy from growth towards the production of secondary metabolites, such as pigments. This survival mechanism prepares the organism for dispersal. [13]Use of Genetic Sequencing for Metabolic Analysis. DNA sequencing (such as that provided by MACROGEN) is a powerful tool for predicting the metabolic pathways and genes involved in the biosynthesis of these metabolites. Once the genome sequence is obtained, bioinformatics tools and databases such as KEGG and MetaCyc can be used to map metabolic pathways and associate them with specific genes. Melanin pathway: Identifying genes for enzymes such as tyrosinase or laccase can indicate that the body is capable of producing melanins. Anthraquinone pathway: Gene sequencing of oxygenases, hydroxylases, and transferases may suggest the ability of the fungus to synthesize anthraquinones. Gene Expression Analysis with DNA-seq. In addition to DNA sequencing, the DNA-seq technique is useful for studying gene expression. It allows us to identify which genes are active under certain conditions, such as nutrient scarcity or exposure to environmental factors that stimulate pigment production. For example, if a fungus such as *Aspergillus* produces melanin in a given environment, the genes related to the melanina they will be expressed in greater amounts, which can be detected in a DNA-seq análisis.[14.15)).Sequence Predictors and Specific Enzymes. Genetic sequencing also makes it possible to predict the existence of specific enzymes involved in the biosynthesis of secondary metabolites. Polyphenol oxidase: In some mushrooms, this enzyme may be involved in the formation of melanins. Their gene can be identified from the genetic sequence. Laccase: This enzyme can also be identified from its gene sequence, and its presence is linked to the production of certain pigments. In many fungi, laccase plays an important role in the formation of dark pigments. The identification of genes that code for laccases from sequencing would be a good indication of the production of pigments such as melanin. In our case, they were not studied.[16] The dehydrogenase pathway plays a crucial role in many metabolic processes within cells, including those related to the production of secondary metabolites. [17] Although not directly linked to the synthesis of pigments such as melanins, the activity of dehydrogenases (enzymes that catalyze dehydrogenation reactions, i.e. the removal of hydrogen atoms) has indirect implications in the generation of secondary metabolites that can influence the coloration of fungi, such as Aspergillus. In general, these dehydrogenases are a group of enzymes that catalyze the oxidation of substrates, which involves the transfer of electrons from a molecule to an electron acceptor, usually NAD^+ or $NADP^+$, converting them into their reduced forms, NADH or NADPH. This process is essential for cellular respiration, compound biosynthesis, and redox reactions of metabolites. Its role in the production of secondary metabolites. In the production of secondary pigments or metabolites in Aspergillus, dehydrogenases may have an indirect but fundamental role, due to their participation in oxidation and reduction reactions that provide precursors or activate metabolic pathways that produce pigments such as melanin or anthraquinones. In general, the dehydrogenase pathway is key to the biosynthesis

of several secondary metabolites, as it contributes to the production of NADH or NADPH, which are essential for redox reactions that transform precursors into final metabolites. [18,19]Although not directly responsible for coloration (like melanins or aflatoxins), dehydrogenases facilitate the oxidation-reduction reactions necessary for the formation of these pigments and other compounds that can affect the coloration of the fungus. In summary, dehydrogenases are essential for activating or boosting the metabolic pathways that lead to pigment synthesis, even though they are not directly responsible for pigmentation. [20,21,22,23,24]

From the analysis of the sequencing of pigment-producing metabolites, 6 pigment-producing metabolites originated by the fungus *Aspergillus salvadorensis* were found, which are:

Table I. Enzymes Reduction of NADPH and NADH Oxidation

NADPH-ferrihemoprotein reductase
NADH-quinone oxidoreductase subunit A [EC:1.6.5.3]
NADH-quinone oxidoreductase subunit B [EC:1.6.5.3]
NADH-quinone oxidoreductase subunit C [EC:1.6.5.3]
NADH-quinone oxidoreductase subunit D [EC:1.6.5.3]
NADH-quinone oxidoreductase subunit F [EC:1.6.5.3]

Fuente: MACROGEN KOREA DEL SUR. EggNOG , MetaCyc . 2025

Table I. This text describes the indirect but crucial role of two enzymes, NADPH-ferrihemoprotein reductase and NADH-quinone oxidoreductase (with its subunits), in the production of pigments in fungi such as *Aspergillus salvadorensis*. Summary of the Enzymatic Role in Pigmentation. The above enzymes do not synthesize pigments directly, but act as essential facilitators by participating in the redox metabolism (oxidation-reduction reactions) of the cell.1. NADPH-ferrihemoprotein reductase. Main Function: Catalyzes the reduction of ferrihemoproteins using NADPH. Pigmentation Implication: Contributes to general metabolic processes that require electron transfer, which is a prerequisite for the biosynthesis of many compounds, including pigments. 2. NADH-quinone oxidoreductase (Subunits A, B, C, D, F). Main Function: Catalyzes the transfer of electrons from NADH to a quinone, being a key part of the electron transport chain for cellular respiration and energy generation. Involvement in Pigmentation: Indirect: Its activity in cellular redox balance is essential for reactions that lead to the synthesis of secondary metabolites, such as melanins and other phenolic compounds (which are usually pigments). Electron Management: They are involved in the oxidation-reduction of intermediate compounds necessary for pigment biosynthesis. Involvement in Pigment Production. The production of dark pigments in the fungus *Aspergillus salvadorensis*, probably melanins, depends on a series of redox reactions. Role of NADH/NADPH: Enzymes are vital because they manage the generation and use of NADH and NADPH. (Bögre, 2003) These compounds are crucial electron transfer cofactors in the formation of products such as melanins. Facilitation of Biosynthesis: In the case of melanin, dehydrogenases (such as NADH-quinone oxidoreductases) help maintain the redox balance that allows the oxidation of phenolic precursors (such as tyrosine) until reaching complex intermediates such as dopaquinone, which eventually form melanin. In essence, these enzymes are vital to the cell's chemical ecosystem, creating the redox environment necessary for direct pigment enzymes to carry out their work.

Table II. Enzymes reduce NADPH and oxidation NADH

NADH-quinone oxidoreductase subunit G
NADH-quinone oxidoreductase subunit H
NADH-quinone oxidoreductase subunit I
Urate oxidase, dihydrolipoamide dehydrogenase
FMN-dependent NADH-azoreductase
COGO431 Nadp dependent fmn reductase
COGO655 Nad dependent fmn reductase
NADP oxidoreductase, coenzyme f420-dependent

Fuente: MACROGEN KOREA DEL SUR. EggNOG , MetaCyc . 2025

Table II. Function and Meaning of NADH-Quinone Oxidoreductase. The enzyme NADH-quinone oxidoreductase (or NADH dehydrogenase), classified as EC:1.6.5.3, is a crucial protein complex. Identification of the Enzyme Complex. The complex is made up of multiple components called subunits (identified by K codes such as A, B, C, D, and F). The fact that all subunits share the same EC number (EC:1.6.5.3) confirms that they are all part of the same complex that catalyzes the same overall reaction. This enzyme is an oxidoreductase, that is, it handles oxidation-reduction reactions, acting specifically on NADH groups and using a quinone as an electron acceptor. Indirect Role in Pigment Production. Although NADH-quinone oxidoreductase is not the enzyme that directly synthesizes pigment, it does play a crucial and indirect role in pigment production, [25]especially in fungi such as *Aspergillus salvadorensis*. Redox Balance. Complex I is the main function of the fungus, being essential for cellular respiration and energy generation. Without it, the cell would not have the energy (ATP) or chemical stability (redox homeostasis) needed to carry out expensive metabolic pathways such as pigment genesis (pigment production). Interaction with Pigment Precursors. In fungi, this enzyme or similar enzymes (such as NADPH-quinone oxidoreductases) can participate in the redox cycle of quinones, which are molecules that act as precursors or byproducts of pigment biosynthesis. There is a direct biochemical interaction: some quinone pigments may even act as oxidants or inhibitors of Complex I, demonstrating a close link between energy metabolism and pigments. In the case of *black A. salvadorensis* (probably melanin), the NADH/NADPH handled by these enzymes is essential for the reduction of intermediate compounds that eventually become those dark pigments. DHN melanin: the black protective shield. The black or gray-green color of *Aspergillus* spores is almost entirely due to DHN-Melanin (1,8-dihydroxynaphthalin-melanin), a final black polymer. Synthesis and Main Function. Composition: DHN-Melanin is a pigment derived from the polyketide pathway, which are chemical precursors. Key Enzyme: The enzyme PksP (Polyketide Synthase) is responsible for initiating the synthesis of the main precursor (a heptacetide). Function: This pigment is deposited on the cell wall of the spores, essentially acting as a protective shield for the survival of the fungus. [26] Stress Induction. The Trigger: The production of melanin is not constant, but is induced (activated) when the fungus is faced with stressful conditions, especially oxidative stress (caused by the accumulation of Reactive Oxygen Species, such as free radicals). Mechanism: The accumulation of acts as a crucial cellular signal, activating genes that allow the fungus to build the melanin defense machinery to survive. The Cellular Energy Connection (NDH). Although the enzyme NADH-quinone oxidoreductase (NDH), or Complex I, does not produce melanin directly, it is critical for synthesis to occur: NDH is essential because it generates the energy (ATP) and maintains the necessary metabolic balance. Without the energy and homeostasis that NDH provides, the enzymatic machinery of DHN-melanin (such as PksP) would not be able to operate or build the pigmented defenses, which could lead to the death of the fungus. In short, stress activates melanin's machinery, but NDH provides it with the fuel to function. [27,28,29,30]

Identification of the molecule of *Aspergillus salvadorensis.*

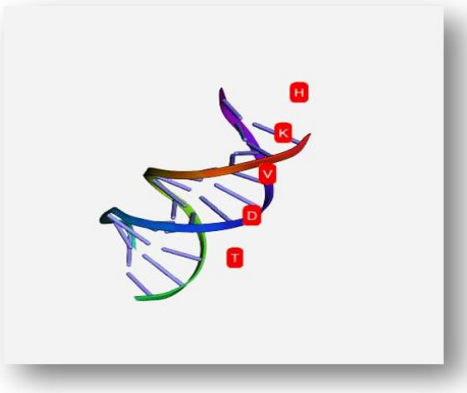

Fig.3. DNA molecule of *Aspergillus salvadorensis.* AI.2025

In Fig 3 you can see Codons (3 bases) and amino acids (one letter) of *Aspergillus salvadorensis*. The image shows a three-dimensional (3D) structure of a nucleic acid, probably DNA or RNA. A double helix (which is typical of double-stranded DNA or RNA) is observed, and a rainbow-colored gradient along the strand, which normally indicates the direction of the molecule (5' to 3'). In addition, there are red letters (T, D, V, K, H) marking certain specific positions of the molecule. The carcinogenic precursors in the fungus are not found in these.

Discussion

The *Aspergillus* genus was first scientifically described in 1729 by Italian biologist Antonio Micheli in his work *Nova Plantarum*. Born in Florence in 1697, Micheli laid the groundwork for understanding this diverse fungal group (31). Today, *Aspergillus* species are of considerable interest across multiple sectors—not only in medical mycology due to their pathogenic potential, but also in the food, chemical, and biotechnology industries for their enzymatic and metabolic capacities.

This study originated from a common and culturally significant plant in the region, the nacascol seed (*Caesalpinia coriaria*), traditionally used in leather tanning and pottery in the northern parts of the country. Notably, the seed serves as a substrate for fungal colonization, particularly by *Aspergillus* species, which metabolize the seed by breaking it down until it is completely pulverized. This degradation process plays a crucial role in dyeing practices: the fermented tannins within the seed yield gallic acid and glucose, while the black

pigmentation of ceramic vessels results from a combination of seed tannin, fungal metabolites, and iron present in the clay. This synthesis gives the pottery its characteristic dark brown to black coloration, contributing to the distinct aesthetic of black clay ceramics.

Ecologically, *Aspergillus* species are saprophytic organisms commonly found on decaying organic matter plant material, seeds, or soil. They exhibit broad thermal adaptability, with growth reported between 5°C and 60°C in many species. Some rare strains can even withstand extreme temperatures ranging from 100°C to 300°C.

Taxonomically, *Aspergillus* is considered an amorphous and complex genus, currently comprising between 260 and 837 species (32, 33). This taxonomic complexity has prompted the integration of modern molecular tools, such as polymerase chain reaction (PCR) and DNA sequencing, for accurate species identification, provided that comprehensive databases and specific primers are available for reliable base-pair matching.

The pigmentation observed in *Aspergillus* and other fungi is attributed to the synthesis of various chromophoric compounds, including melanins, carotenoids, flavins, phenazines, and quinones (34). Melanin, in particular, plays a protective role for spores, guarding against environmental stressors such as temperature fluctuations, UV radiation, and desiccation (35, 36). It is present in nearly all pathogenic fungi and contributes to fungal survival and virulence.

In *Aspergillus niger*, melanins are composed of both aliphatic and aromatic molecules, often derived from indole or phenolic precursors. These pigments are classified as secondary metabolites and display significant variability in structure and function. Nonetheless, they share common physicochemical properties, including resistance to acid hydrolysis, amorphous and polydisperse structures, negative surface charge, polymeric configuration, and a stable free radical system (37). Except for pyomelanin, most melanins are insoluble in water and organic solvents, dissolving only under alkaline conditions. These unique chemical traits contribute to the ecological adaptability of so-called "black fungi", enabling them to thrive in diverse and sometimes extreme environments (27, 35).

Conclusions

This research identified 14 enzymes and secondary metabolites associated with the biosynthesis of black pigments in *Aspergillus* species, particularly under conditions of oxidative stress. These include:

i. NADPH-ferriheme protein reductase
ii. NADPH-quinone oxidoreductase subunits A, B, C, D, F
iii. NADH-quinone oxidoreductase subunits G, H, I
iv. Urate oxidase
v. Dihydrolipoamide dehydrogenase (FMN-dependent)
vi. NADH-azoreductase
vii. NADP-dependent FMN reductases (COGO431, COGO655)
viii. NADP oxidoreductase
ix. Coenzyme F420-dependent oxidoreductase

These enzymatic components are involved in redox reactions and are upregulated as a cellular response to oxidative stress. Their activity contributes directly to the biosynthesis of melanins and other dark pigments, reinforcing the role of *Aspergillus* not just as a decomposer, but also as a biochemical agent with significant industrial and ecological relevance.

Abbreviations

DNA	Deoxyribonucleic Acid
UES	University of El Salvador
CENSALUD	Center for Health Research
FASTA	Format for Nucleotide Sequences
MACROGEN	Macroscopic Phenotype of Gene
MERK	Merck Sharp and Dohme
NGS	Sanger's Sequencing Techniques
PCR	The polymerase Chain Reaction
SMRT	Single Molecule, Real-time
SMSR	Shotgun Metagenome Sequencing Report
SBS	Sequencing by Synthesis
TGS	Third Generation Sequencing

Gratitude and recognition

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Contributions from authors

This work was solely authored by Antonio Vásquez Hidalgo, who has reviewed and approved the final version of the manuscript.

Conflict of Interest Statement

The author declares that there are no conflicts of interest regarding the publication of this study.

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