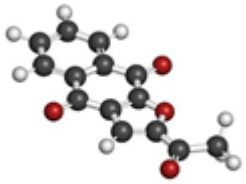

Presence of enzymes and secondary metabolites clusters in DNA sequence of *Aspergillus salvadorensis* in the production of natural black pigments.[1]

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Resumen

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: Database offering detailed information on metabolic pathways, enzymes, compounds, and reactions, UniRef: The UniProt Reference Clusters (UniRef) offer clustered sets of sequences derived from the UniProt Knowledgebase, including isoforms, and selected UniParc records, 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 and enzymes.

Introduction

In our environment it is not common for a fungus to produce black pigmentation with the presence of the nacascol seed, it has an inert particularity, it needs the presence of a fungus of ***Aspergillus salvadorensis***, which in contact with the tannin of the seed gives the characteristic black color or produce the dye by its own means. The seed comes from the tree known as nacascol and has the scientific name ***Caeselpinia coriaria***. It belongs to the *Caesalpnieaceae family*, of the genus *Caesalpinia* and *a coriaria* species because it is used in the dyeing of cattle skin hides. It is a leguminous plant with a stem 3 to 11 meters high, with leaves in pinnae pairs 5 to 10 cm long (each with more than 10 leaflets 4 to 8 mm long and 2 mm wide, rounded apex) and seeds of Black brown color. In these seeds grows a fungus called ***Aspergillus salvadorensis***, its spores are arranged in the form of separate spicules throughout the spore. They are multiple, coalescing, pigmented, small in diameter,

and black in color. When grown in a special medium, they generate a black coloration with pigment production in the posterior region of the tube before 24 hours, by adding more oxidizing substances to the flavoror agar medium, it gives the characteristic color of black color when interacting with the iron or by themselves generating the pigment since it contains secondary metabolites. According to the preliminary phytochemical analysis of the *Caeselpinia coriaria tree*, it contains tannins, triterpenes, glycosides, and flavonoids. **Justification**. Initially, a complete study was carried out on the extraction and identification of DNA from the genus *Aspergillus* to determine what type of species circulate in our country. The research began in 2006 with phenotype studies in the laboratories of the Microbiology department of the Faculty of Medicine of the University of El Salvador, then in 2008 in the scanning electron microscopy laboratories at CENSALUD and then in Mexico preliminary results of molecular PCR, then in 2024 in the laboratories of MACROGEN INC in South Korea Illumina method the complete extraction study, quantification and sequencing of g,q,c,ds,seqDNA in its molecular analysis of the genus *Aspergillus* to determine the type of native species circulating in the northern part of our 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:** 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.

The research was composed of four phases: First phase: Collection of nacascol seeds. Five batches of *Caeselpinia coriaria* seeds were collected from the northern zone, purification and selection of the seeds according to their appearance and color. The inoculums were then inoculated into plates and test tubes in Sabouraud culture medium. Growth was observed at 48 hours in the Microbiology laboratory at 40x and 100x under a simple light microscope over weeks with a comparison sheet. Second phase: Extraction and preparation of the tannin from the seed and the fungus. Preparation of the spore suspension and microculture. A spore suspension was prepared from culture where the conidia or spores were identified where they were extracted by simple method under the microscope and reseeded, which was used to perform the inoculum in the Sabouraud culture medium in tube and plate, the fungus was sown in a 250 ml Erlenmeyer flask, to which 50 ml of the medium was previously added, incubated for 48 hrs at 28 ºC. Subsequently, 100 ml of distilled water at room temperature was added and carefully stirred for 5 min with the help of a magnetic stirrer. It was also passed into the tube in Saboraud and stirred for

homogenization to ensure that the spores are well suspended. If the spores tend to settle, it may be helpful to use a mixer or shake manually. Spore counting was performed with a Neubauer chamber for adequate concentration required. [1]). A micro culture was carried out by preparing in a sterile wet chamber: a petri dish, at the bottom of which sterile water is placed and a V-shaped glass rod, on which the slide is placed, and on top of it the block of the Saboraud culture medium. The sample was inoculated on the block in four quadrants, by means of the L-shaped platinum loop on the block of the medium already inoculated, a coverslip sheet is placed and incubated for 7 days at 25°C. Once the incubation period is over, the coverslip is removed and placed on a sheet with one or two drops of Lactophenol Blue and the characteristic structures of the isolated species are observed under a microscope with a 100x objective. [2] The fungus was isolated from nacascol seeds from the northern zone. The brown-black seeds were identified, collected and stored in airtight bags for later analysis at room temperature, being mostly black. It does not require special measures for transport and conservation. Seed cleaning was not used in the plant material. It was scraped and the material was removed and deposited in ASD (Sabouraud dextrose agar) culture media for isolation and differentiation. Third phase: Laboratory tests, which consisted of sowing the inoculum of the fungus present in the seed in sabouroud agar tubes for cultivation. The culture plates and tubes were incubated at temperature for one week. This process was repeated until pure isolates were obtained. Previously isolated samples were inoculated in 150 mL of PW liquid culture medium (peptone, water, and nutrient broth) in dish and tubes and incubated at 37 °C in 48 hours and at room temperature between 20 and 25 °C. The biomass was dried at 42 °C for two days and left at room temperature at 25 °C for 3 weeks until further analysis. **Morphological characterization of fungal samples**. It was performed in cultures in Saboraud and incubated at 37°C for 72 hours in aerobic tubes and then at room temperature x 7 days. With simple microscopic observation on cotton blue lactophenol at 10 x, 40 x and 100x, and then sealed with a coverslip. The visualization of the structures was carried out under an optical microscope. Taxonomic affiliation was used to determine the online database. DNA method. In general, methods for DNA sequencing were used. In the fourth phase, molecular characterization. The secondary metabolites found in the DNA chain, provided by MACROGEN INC 2025 South Korea, were identified by sequencing, as well as the result of enzymes, proteins and secondary metabolites of the fungus *A. salvadorensis*. The preparation consisted of three samples, a pure strain in a glass tube and two more samples, one sample was DNA and the other simple, were sent to South Korea in the laboratories of MACROGEN INC for the extraction, analysis and identification of g,q,c,ds,seqDNA. The Sanger sequencing method was used using the enzyme chain termination method and automatic sequencing. Sequencing is carried out in three steps: 1. Perform the synthesis of new DNA fragments. 2. Separate the fragments by electrophoresis and finally 3. Identify nucleotides to determine the sequence. In terms of microbiology, molecular study can be performed by sequencing small fragments of deoxyribonucleic acid (DNA) that have been previously amplified, or by sequencing all of the randomly fragmented previously fragmented DNA from the

fungus. Sequencing through *Illumina* is basically characterized by the execution of the following processes: a. The amplification of DNA fragments for the generation of clusters (colonies of the same fragment) is carried out using the PCR bridge method. b. Base detection in sequencing is done through fluorescent markers. Therefore, the Illumina platform allows for the sequencing of DNA fragments at both ends. The fragment is sequenced amplified by both ends rather than a single end. Including gDNA. qDNA, dsDNA, cDNA, and seqDNA and then analyze them in high-capacity computer packages. [(3)] 6. gDNA extraction. gDNA genes are currently one of the most studied forms in the fungal genome, mainly for the detection and identification of species through molecular biology. In the preparation of the above sample and its extraction method, its main objective is that it must release the intracellular DNA, breaking the wall of the fungal cell or mycelium, the cell membrane and the nuclear membrane by heat at a temperature controlled by the thermal cycler or another pathway. In addition, it must concentrate, DNA target molecules that may be present in small quantities and must be purified by removing traces of contaminants, proteins, foreign RNA remains, without degrading nucleic acid. For the rupture of fungal cells it can be performed by different methods: such as chemical, enzymatic or mechanical. [(4)]), which are the most common, but it will depend on the lab in its extraction method. [(3)]7. qPCR technique. Called quantitative DNA measured by fluorometry that captures nucleotides. After having met the requirements for gDNA extraction in quality control, the cDNA was fragmented and quantified by degrading it. The DNA quality control method in MACROGEN was: 1. Quantity of DNA: Performed by the QuantiFluor® dsDNA System method using the Victor Nivo multi-mode microplate reader. Macrogen quantifies the starting genomic material using fluorescence-based quantification. using specific double-stranded DNA dyes, this method quantifies dsDNA more accurately than the UV spectrometer, even in the presence of common contaminants. The species of the genus *Aspergillus spp* in their genomic evolution have been variable in their size, in which they have an average genome size range of 27 to 39 Mb or more and an average number of genes between 9000 to 14000 genes. The one with the largest size is *A. sojae* 39.5 MB and the one with the highest number of genes is *A. niger* with 14,165 MB. [5-8] 8. Quality per cycle. The term Cycle Quality refers to the evaluation of the quality of sequencing readings based on each sequencing cycle. The sequencing process is carried out in cycles. During each cycle, a base is added to the DNA fragment and the embedded base is detected. It is sequenced base by base in a series of cycles. In each cycle, a base (A, T, C, or G) is incorporated into the DNA strand, and a fluorophore or optical signal detects the aggregated base. This process is repeated several times, and in each cycle it is detected and recorded in a specific way. For the identification of cluster genes in enzymes, proteins and secondary metabolites, MACROGEN used the following systems: the EggNOG summary: Orthology Frequency within COG (Clusters of Orthologous Groups) Categories in the sequencing reading. MetaCyc: Database offering detailed information on metabolic pathways, enzymes, compounds, and reactions, UniRef: The UniProt Reference Clusters (UniRef) offer clustered sets of sequences derived from the UniProt Knowledgebase,

including isoforms, and selected UniParc records, 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.

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 pigmented in color, height of the mycelium low, appearance of the colony is dusty moist black. **Microscopic characteristics**: Sterygmas. The head of the conidia is black, conidiophore rough 1 to 4 mm long with conidia or abundant internal spores of 1 to 3 microns, colored brown to black, the conidial heads smooth with a round and irregular wall, arranged in the form of uniserial chains; smooth, pronounced thin-walled tips, colored brown to black; the gallbladder or columella; there are abundant conidia; it has a row of phylalides from which the conidia emerge. The sporangium is a simple peridial globose structure, of the esterigma it is black in color. The mature brown conidia are spherical with projections in the form of triangular peaks throughout the periphery, abundant; and the central ones are scarce forming spicules in number greater than ten. The conidia has a spherical stellate appearance with spicules, from the ends emerge filaments arranged in the form of linear chains that come out of the protuberances. The entire structure forms a solid shell that is not dyed with cotton blue lactophenol. The immature asexual spores are spherical, thin-walled, colorless, large in size that are found in the conidiophore in which they are close to the foot, they are immature and then eventually reach the vesicle, they are mature and under pressure they are expelled outwards in the phylalides.

	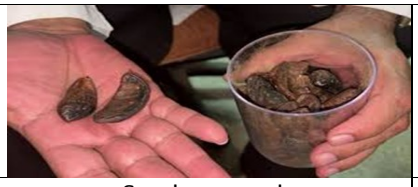	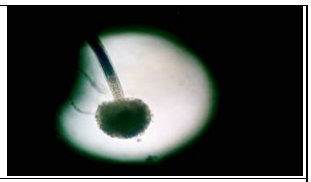
Seed nacascol	Seed nacascol	100x A.salvadorensis
		
Sabouraud Agar culture	Sabouraud Agar culture reverse	tube Agar reverse
Photo 1. Seed & culture Agar *Aspergillus salvadorensis*	Laboratory Microbiology UES	

Photo 1. 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. (Raw Data Statistics.). The section consists of: A. Experimental workflow. Macrogen**.** Samples are prepared according to the NGS library preparation workflow and sequenced using the Illumina platform. The process may differ depending on the library preparation protocol followed. For example**:** Sample preparation. DNA/RNA is first extracted from the sample, and samples that meet quality control standards proceed to the construction of the library. Ligate adapters. The sequencing library is prepared by random fragmentation of the DNA or cDNA sample, followed by a 5' and 3' adapter ligation. Alternatively, marking combines the fragmentation and ligature reactions in a single step, greatly increasing the efficiency of the library preparation process. Final construction of the library. The adapter-bound fragments are then amplified by PCR with a PCR primer solution that is annealed at the ends of each adapter. Library templates undergo a quality control and quantification process. Cluster generation through bridge amplification. The library is loaded into a flow cell where fragments are captured on a lawn of surface-bound oligonucleotides complementary to the library adapters. Each fragment is then amplified into different clonal groups through bridging amplification. After cluster generation is complete, the templates are ready for sequencing. Synthesis Sequencing (SBS) technology. Illumina's SBS technology uses a patented reversible terminator-based method that detects individual bases as they are incorporated into DNA strands of moldings. Since all terminator-bound reversible dNTP 4s are present during each sequencing cycle, natural competition minimizes incorporation bias and greatly reduces gross error rates compared to other technologies. The result is high-precision base-by-base sequencing that virtually eliminates sequence context-specific errors, even within regions of repeating sequence and homopolymers. Chemistry of four, two and one channel. Systems using four-channel chemistry use a mixture of nucleotides labeled with four different fluorescent dyes. Similarly, two-channel chemistry uses two different fluorescent dyes, and one-channel chemistry uses only one dye. The images are processed by image analysis software to

determine the identity of the nucleotides. Raw data generation. The Illumina sequencer generates raw images using sequencing control software for system control and base calling, through an integrated primary analysis software called RTA (Real-Time Analysis). BCL/cBCL binaries (called base) are converted to FASTQ files using bcl-convert, which is a package provided by Illumina. The adapters are not trimmed away from the readings.

Identified sequencing metabolites.

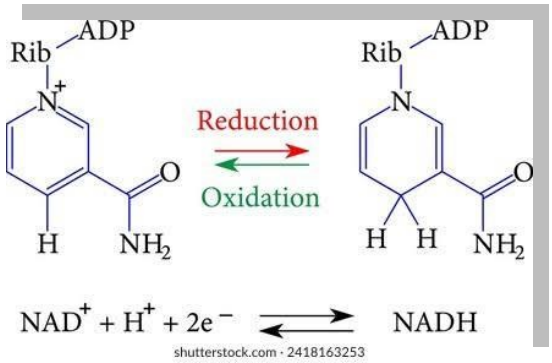

Fig 2. NADH molecule.
Source: Taken from Shutterstock with the ID 2418163253.free 2025

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.*

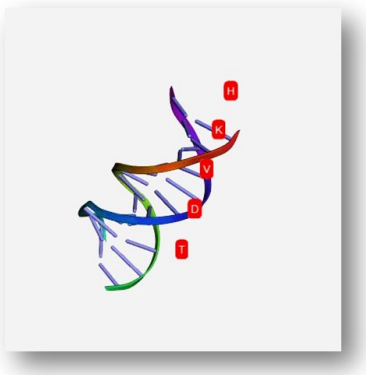

Fig.3. DNA molecule of *Aspergillus salvadorensis.* AI.2025

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 initial study of the *Aspergillus* fungus was first described in 1729 by the Italian biologist **Antonio Micheli** in his work Nova Plantarum who was born in Florence, Italy, in 1697 [(31).] Today, it has generated a lot of interest not only because of its implications on health but also in the food, chemical and biological industries. General research began on a natural plant well known in our environment at the national level known as the Nacascol seed, widely used in pottery and leather tanning in the northern part of the country. This seed has an inert particularity that requires the presence of a fungus of the genus *Aspergillus sp* and the fungus devours the seed as a food substrate by piercing it until it is pulverized, so that it can be dyed together with the properties of the clay dyes the vessels black-brown and tanning the skin of the cattle, The tannins when fermented can extract gallic acid and glucose**.** In addition, the vessels give the special characteristic of black clay, which gives a colonial and beautiful look to the vessels. When looking at the pots, the brown tone is from the tannin of the seed and the black comes from the mushroom plus iron contained in the clay gives it the intense black tone. In general, species of the genus *Aspergillus* are found as saprophytes deposited in organic material such as plants, seeds or soil. Their temperature is adaptive, from a minimum of 5 ºC to 60 ºC in other species, although rare they withstand temperatures of more than 100 to 300 ºC. *Aspergillus* is currently an amorphous genus, comprising between 260 and 837 species [(32,33)] Such is the

diversity of species that with the use of modern technologies such as PCR. Sequencing has made it possible to facilitate its study in some cases, as long as an adequate sequential database is available and the primers according to species are available to mix the base pairs. The pigmentation of fungi is due to the synthesis of various types of pigments and chromophore molecules such as carotenoids, melanins, flavins, phenazines, quinones among others. [34]. Melanin pigments have a primary role in protecting fungal spores against temperature increases, radiation, and desiccation. [35] Melanins are present in most fungi, being found in almost all pathogenic fungi. [36] The natural habitat of *Aspergillus* species is hay, seeds and compost, used as feed for cattle. In the case of *Aspergillus niger*. Melanins are composed of aliphatic and aromatic structures of the indole or phenol type and are synthesized by a wide range of organisms.These pigments, which are secondary metabolites, can have different coloration and can also be heterogeneous in terms of their structural organization, composition, and function. However, all of these pigments have similar psychochemical properties, including resistance to acid hydrolysis, amorphous and polydisperse structure, polymeric nature, net negative charge, and a stable radical structure [37]. All melanins, except pyomelanin, are insoluble in water and organic solvents, being only solubilized in alkaline solvents. These chemical properties explain the multifunctional roles of dark pigments that allow black fungi to adapt to various environmental conditions. [27,35]

Conclusions

14 enzymes and secondary metabolites were found in the production of black pigments produced by the fungus by oxidative stress. Among them: NADPH-ferrilhemoprotein reductase,NADPH-quinone oxidoreductase subunit A,NADPH-quinone oxidoreductase subunit B,NADPH-quinone oxidoreductase subunit C,NADPH-quinone oxidoreductase subunit D,NADPH-quinone oxidoreductase subunit F, NADH-quinone oxidoreductase subunit G, NADH-quinone oxidoreductase subunit H,NADH-quinone oxidoreductase subunitI, Urate oxidase, dihydrolipoamide dehydrogenase,FMN-dependent, NADH-azoreductase, COGO431 Nadp dependent fmn reductase, COGO655, Nad dependent fmn reductase ,NADP oxidoreductase, coenzyme f420-dependent. They produce it when there is oxidative stress.

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

Antonio Vásquez Hidalgo is the only author. The author read and approved the final manuscript.

Conflicts of interest

The author declares that he has no conflicts of interest.

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