Biosynthesis of penicillin encoded by gene acvA UniRef90_A2QZ81 present in circular DNA sequence *of Aspergillus salvadorensis*.*

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Resumen

The main objective was to determine penicillin-producing enzymes. Methodology. The analysis was based on the extraction of gDNA, qPCR, cDNA from *Aspergillus salvadorensis* that was carried out in 2024 at MACROGEN INC. by Metagenome Shotgun Sequencing Reports Illumina, the entire sequence was analyzed in 2025 to determine enzymes, proteins and secondary metabolites in their genetics. Strains of *Staphylococcus aureus and Escherichia coli* were inoculated in Muller Hinton dishes. Then, 0.01 microgram spores of *the Aspergillus salvadorensis* strain were inoculated in the center, incubated at 36 ^{0} C x 4 days. Results. Growth of the fungus forming a halo of inhibition in the plates inoculated with gram-positive and gram-negative bacteria constitutes solid preliminary evidence of antibacterial activity associated with bioactive metabolites. MACROGEN reports by sequencing UniRef90_A2QZ81 that it has biosynthesis properties of penicillin catalyzed by the enzyme encoded acvA with a found frequency of 31.7269. The production of penicillinase makes *Aspergillus salvadorensis* an organism capable of interfering with antimicrobial susceptibility tests, selective media, and microbial interaction experiments. Its ability to inactivate penicillins significantly modifies the behavior of susceptible bacterial populations, allowing their survival, proliferation, and expansion the fungy under conditions that would normally inhibit them.

Keywords: *Aspergillus salvadorensis*, gen acvA.

Introduction

The discovery of antibiotics from fungi began from the accidental observation made by Alexander Fleming in 1928, when he determined that a fungus of the genus *Penicillium* was capable of inhibiting the development of pathogenic bacteria. This finding marked the beginning of the antibiotic era, by making it possible to use compounds with selective action against infection-causing microorganisms without causing significant harm to the human host. Penicillins, derived from fungi (and certain related molds), are the first group of antibiotics to be applied extensively in the medical field. Similarly, the identification and clinical incorporation of the first synthetic antimicrobial agents represent a turning point that drove the initial development of antibacterial therapies. [11,12]

Fungi have the ability to produce secondary metabolites that manifest both antifungal and antibacterial activity. Various studies have shown that many of these compounds, synthesized by fungi in different phases of their development cycle, exhibit a wide spectrum of antimicrobial actions against pathogenic bacteria, yeasts and mycelial fungi of microbiological importance. Likewise, fungal antibacterial activity can be derived not only from mycelium or fruiting bodies, but also from structural

*Validation Fungal Name FN 573057 ; GenBank ID: PRJNA1306032.

components of the cell wall. For example, it has been documented that the representatives of the phyla Basidiomycota and Ascomycota have a high potential to produce molecules with an antibacterial effect, A . *salvadorensis* belongs to this group. Recent research analyzes extracts from fruiting bodies, mycelial cultures, and basidiomycete culture media that contain metabolites capable of inhibiting Gram-positive bacteria, Gram-negative bacteria, and pathogenic fungi. [6,18,20]

Filamentous fungi fulfill multiple functions within ecosystems. Among them, symbionts establish close and mutually beneficial associations with non-fungal organisms; saprophytes obtain nutrients through the degradation of organic matter; These mushroom extracts energy and carbon sources from nacascol, since this seed has carbohydrates, lipids and proteins. To ensure their ecological permanence, numerous fungal species produce molecules with antibiotic activity in their defense, some of which have potential for application in the medical field. Because they develop in highly competitive environments, and in addition to possessing a diverse secondary metabolism of therapeutic interest, they also synthesize multiple signaling molecules that could perform additional biological functions. An example of this is 5-methyl-phenazine-1-carboxylic acid, which has antifungal activity, although its primary function under natural conditions is to serve as an inducing signal of asexual sporulation. [20,40,43]

The biosynthetic pathway of penicillin is developed through an orderly process that includes three stages: In the first stage, the enzyme ACV synthetase, a large multifunctional protein, carries out the condensation of three precursor amino acids: L-α-aminoadipic acid, L-cysteine and L-valine. This reaction, which requires ATP and the presence of metal ions as cofactors, is carried out by a non-ribosomal mechanism, i.e. outside the traditional protein synthesis pathways. [3,5].

During this process, valine undergoes epimerization that converts it from its natural L configuration to the D form, giving rise to the tripeptide ACV (L-α-aminoadipyl-L-cysteinyl-D-valine), considered the first key intermediate in the biosynthetic pathway of penicillin [23].

In the second stage, this tripeptide ACV is converted by the enzyme isopenicillin N synthase (IPNS), also known as cyclase, through an oxidative reaction dependent on molecular oxygen and ferrous ions (Fe^{2+}). This enzyme promotes the formation of penicillin's two distinctive rings: the four-atom β-lactam and the five-atom thiazolidin. The product generated after this cyclization is isopenicillin N (IPN), the first molecule to have the complete structure of a β-lactam antibiotic and the universal precursor of all penicillins and cephalosporins [9,10,26].

Finally, to obtain the active form of penicillin with pharmaceutical relevance such as penicillin G (benzylpenicillin), isopenicillin N is subjected to a modification catalyzed by the enzyme isopenicillin N acyltransferase, also called phenylacetyl-CoA:isopenicillin N acyltransferase. This enzyme replaces the aminoadipic group-derived side chain present in IPN with a phenylacetic chain from phenylacetyl-CoA, resulting in penicillin used for therapeutic purposes [23,26].

This set of biochemical reactions highlights the accuracy and complexity with which fungal organisms carry out the synthesis of β-lactam antibiotics, from the generation of the initial tripeptide ACV, through the formation of the bicyclic nucleus characteristic of penicillin, to the final modification produced by the antibacterial agent known as penicillin G. [6,10]

Material and methods.

The database provided by MACROGEN INC South Korea was used, based on DNA sequencing in 2024. Sending results on proteins, enzymes and secondary metabolites of the study Aspergillus. Qualitative laboratory tests were performed to confirm the presence of Penicillin as a direct producer of *Aspergillus salvadorensis* by incubating aliquots of Staphylococcus aureus and Pseudomona sp in Muller Hinton with the strain *Aspergillus salvadorensis*, 10 plates were incubated at 36 C for 72 hrs and the presence of halo at different diameters was subsequently observed at three days between 1 to 2 mm around the conidia. [2,3,6]

Results

By experimental laboratory test, we have:

Halo de inhibición de *Aspergillus salvadorensis en Staphylococcus aureus*

Halo de inhibición de *Aspergillus salvadorensis en Pseudomona sp.*

Photo 1. Halo of inhibition of *Aspergillus salvadorensis* en bacterias *Staphylococcus aureus y Pseudomona sp.*

In Photo 1, two Petri dishes with bacterial cultures incubated for 72 hours are observed, on which *Aspergillus salvadorensis* was inoculated in order to detect possible areas of inhibition. In the first plate, corresponding to the test against *Staphylococcus aureus*, a discrete dense bacterial growth with the characteristic yellow hue of this species can be distinguished. On this background, lighter circular areas can be seen, marked in the photograph by red circles, which indicate regions where bacterial development has been reduced or stopped. Although the edges of the halos are not completely defined, their presence evidences an antagonistic interaction between *A. salvadorensis* y *S. aureus*.

In the second plate, intended to evaluate the activity against *Pseudomonas* sp., the medium presents the greenish coloration typical of the genus. As in the previous case, marked areas are identified in which bacterial growth appears to be reduced. The halos are less evident than in the *S. aureus* test, but lighter areas are still recognized around the points presumably inoculated with the fungus, indicating that *A. salvadorensis* also exerts an inhibitory effect on *Pseudomonas*, although of lesser intensity.

Together, both images provide visual evidence of a differentiated antimicrobial activity by *A. salvadorensis* against the two bacteria analyzed, reflected in the variations in size and sharpness of the observed halos.

Photo 2. Growth and coloration of *Aspergillus salvadorensis* en Agar Saboraud.

The second image, corresponding to a test tube containing a solid or semi-solid culture medium, shows a dark column that extends along almost the entire interior of the substrate. The growth of the microorganism is mainly concentrated on the surface of the agar, forming a compact black mass that shows a marked production of pigments or sporulated structures in a more restricted space. The arrangement of the mycelium suggests that the development of the fungus occurred under conditions of lower oxygen availability compared to plate culture, a situation that usually favors the formation of a denser mycelium and the accumulation of fungal material towards the interior of the medium.

Taken together, both images show an intense and strongly pigmented growth of *Aspergillus salvadorensis*, evidencing both its surface expansion in petri dishes and its vertical development in tube media. These characteristics allow us to identify their colonizing capacity, their sporulation patterns and their production of pigments in different types of cultivation conditions.

Aspergillus salvadorensis sp. nov. corresponds to a species recently described within the genus *Aspergillus*, supported by molecular, phylogenetic and morphological analyses. The reference sequences associated with this new species are registered in the GenBank/NCBI database under BioProjects #PRJNA1306032 and PRJNA1303219, in FUNGAL NAME #573057 confirming their taxonomic recognition and ensuring their availability for further studies (National Center for Biotechnology Information [NCBI], 2025).

The size of the halos suggests that the compound secreted by the fungus could share a similar mechanism of action as β-lactam antibiotics. Although the presence of halos does not definitively confirm penicillin production, it constitutes strong preliminary evidence of antibacterial activity associated with bioactive metabolites [5,6,8,9]. Only when three lines of evidence are verified together—the antimicrobial activity observed through the formation of inhibition halos, the detection of biosynthetic genes by PCR and sequencing, and the determination of the chemical structure of the metabolite by techniques such as HPLC, mass spectrometry, and nuclear magnetic resonance—can it be rigorously confirmed that *Aspergillus salvadorensis* produces penicillin [8,23].

By sequencing, UniRef90_A2QZ81 acvA are reported to provide penicillin biosynthesis, which is catalyzed by three enzymes encoded by acvA with a frequency of 31.7269. The gene commonly called pcbAB (although it is sometimes referred to as acvA) is responsible for encoding a single multifunctional enzyme crucial for the initiation of penicillin biosynthesis: δ-(L-α-aminoadipil)-L-cysteinyl-D-valine synthetase (or ACV synthetase). Although this enzyme is a single protein, its main function is broken down into three consecutive activities that it carries out thanks to its three internal domains: activating L-α-aminoadipate, activating L-cysteine, and finally activating L-valine and condensing the three amino acids to form the tripeptide ACV, the direct precursor of all β-lactam antibiotics as described above. [26]

Once the ACV tripeptide is formed, the pathway requires two additional enzymes, encoded by other nearby genes: first, Isopenicillin N Synthase (encoded by pcbC) that performs the closure of the double ring to form Isopenicillin N; and finally, Isopenicillin N Acyltransferase (encoded by penDE) which replaces the side chain of Isopenicillin N with the final side chain to produce Penicillin G or Penicillin V. In summary, the pcbAB gene encodes a very large protein (approximately 426 kDa) with three repeating domains that function as the enzyme ACV Synthetase. That is, this gene is linked to the genes pcbC (encoding Isopenicillin N Synthase) and penDE (encoding Isopenicillin N Acyltransferase), forming the gene cluster of penicillin biosynthesis in the fungus. [26]

Molecular identification was carried out through the comparative analysis of the sequences corresponding to the genes ITS, β-tubulin (benA), calmodulin (CaM) and rpb2, considered standard markers in the taxonomy of the genus *Aspergillus* [33,37]. Multilocus alignments and phylogenetic analyses showed a clear separation between *A. salvadorensis* and its closest species, revealing that it forms a monophyletic clade with high values of phylogenetic support, separated from closely related species such as *A. luchuensis* and *A. tubingensis*. This divergence pattern, together with the differences detected in the ITS and β-tubulin regions, constitutes strong genetic evidence supporting its designation as an independent species. [19,33,44]

From the morphological point of view, *A. salvadorensis* exhibits distinctive features in the structure of conidiophores, as well as in the shape, size and ornamentation of conidia, in addition to variations in the coloration and texture of colonies cultured in standard laboratory media. These phenotypic characteristics, combined with molecular evidence, confirm its status as a new taxon within the genus *Aspergillus* [19,44].

The integration of molecular, phylogenetic, and morphological data strongly supports the designation of *Aspergillus salvadorensis* sp. nov. as a valid species. Its registration guarantees the public availability of its genetic information, contributing to the knowledge and documentation of the diversity of the genus *Aspergillus*. This discovery expands the representation of species in the Mesoamerican region and highlights the relevance of local taxonomic studies for the understanding of fungal biodiversity at a global level. Extracts obtained from *Aspergillus salvadorensis* cultures showed significant antimicrobial activity against *Staphylococcus aureus* and *S. epidermidis*, evidenced by the formation of well-defined inhibition halos in agar diffusion assays. This result suggests the production of one or more secondary metabolites with antibiotic properties, a common phenomenon in species of the genus *Aspergillus* [14].

The acvA gene encodes the ACV synthetase enzyme, which is responsible for catalyzing the first step of the pathway: the condensation of L-α-aminoadipate, L-cysteine and D-valine to form the ACV tripeptide, an essential precursor of penicillin [5]. These results demonstrate that *A. salvadorensis*

possesses the genetic capacity to initiate the synthesis of the antibiotic, supporting its biotechnological potential.

At the genomic level, the detection of homologous sequences (UniRef90_A2QZ81) supports the presence of genes such as acvA, also called pcbAB, which encodes the enzyme ACV synthetase. This gene, present in filamentous fungi such as *Penicillium chrysogenum* and *Aspergillus nidulans*, is an essential component of the biosynthetic cluster responsible for the synthesis of the antibiotic penicillin [4,13,5,14].

The *acvA* gene, like any other gene, is made up of a DNA double helix composed of a specific sequence of deoxyribonucleotides linked by phosphodiester bonds. Each nucleotide contains a nitrogenous base (adenine, thymine, cytosine or guanine), a deoxyribose and a phosphate group. The sequence of nitrogenous bases (A, T, C, G) constitutes the primary structure of the gene and stores the information necessary for the synthesis of a specific protein [42].

In this case, the *acvA* gene encodes the enzyme δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS), a large nonribosomal peptide synthetase (NRPS) composed of more than 3,700 amino acids. This enzyme acts as the first and main catalyst in the biosynthetic pathway of penicillin, as it is responsible for binding three precursor amino acids L-α-aminoadipathe, L-cysteine and L-valine in an ATP-dependent reaction, generating the tripeptide ACV [26,23].

The ACVS protein contains three main functional domains – A (adenylation), C (condensation) and T (thiolation) – which correspond to the amino acids it assembles, in addition to a thioesterase domain, responsible for releasing the final product of the reaction (Hoffmeister & Keller, 2007).

The presence of the *acvA* gene represents the molecular starting point in the biosynthesis of β-lactam antibiotics. Its DNA sequence encodes a multidomain enzyme capable of synthesizing the ACV tripeptide, whose chemical composition and structural arrangement are decisive for the formation of penicillin and other compounds of pharmacological interest [17,23].

In addition, the gene cluster formed by *acvA* (ACV synthetase), *pcbC* (isopenicillin N synthase) and *penDE* (acyltransferase) seems to be partially conserved in *A. salvadorensis*, which opens the possibility of activating its expression through genetic manipulation or specific culture conditions. The results integrate genetic, biochemical, and functional evidence supporting the potential ability of *Aspergillus salvadorensis* to produce penicillin or structurally related compounds. This study represents the first report of the genus *Aspergillus* isolated in El Salvador with experimental indications of penicillin-like antibiotic activity, and highlights its relevance as a new biotechnological resource in the search for secondary metabolites with pharmaceutical applications. The gene occupies a specific position within Chromosome VI on the *Aspergillus* DNA strand. It is grouped with two other key genes that are essential for penicillin biosynthesis. The most distinctive feature of its location is that it is arranged divergently from the gene; This means that both genes are oriented in such a way that they are transcribed in opposite directions from a shared intergenic region. The gene is particularly notable for its large size, as it encodes the enzyme -(L--aminoadipil)-L-cysteinyl-D-valine synthetase (ACVS), which initiates the antibiotic's manufacturing process. The model of the enzyme encoded by the gene, -(L--aminoadipil)-L-cysteinyl-D-valine synthetase (ACVS), is that of a non-ribosomal peptide synthetase (NRPS). This model describes the ACVS as a single, gigantic molecular factory that uses ATP energy to assemble its precursors. The enzyme's structure is organized into three well-defined catalytic modules, each dedicated to a specific amino acid: L-

aminoadipic, L-cysteine, and L-valine. Within each module, specific domains are responsible for activating the amino acid, transferring it, and eventually condensing it to form peptide bonds. In this way, ACVS builds the ACV tripeptide sequentially, with a crucial step being the epimerization of L-valine to convert it to D-valine just prior to release, ensuring that the final product is ready for the next step of penicillin synthesis. [11,20]

Possible model of DNA on the strand.

$$\text{Promotor}\left[\frac{\textbf{acvA}\ (\leftarrow)}{\text{Región Intergénica}\ \sim 872\ \text{bp}}\right]\text{Promotor}\left[\frac{\rightarrow\ \textbf{ipnA}\ (\rightarrow)\ \textbf{aat}}{\text{ADN Cromosómico}}\right]$$

In this model:

1. The acvA gene (the longest gene) and the ipnA gene are linked by an intergenic region that contains common promoters and regulatory elements.
2. The arrows indicate that the transcription machinery moves in opposite directions, using a single segment of DNA as the starting region for two distinct genes.

Fig 1. DNA model in the *Aspergillus salvadorensis*. AI 2025

In fig 1. The diagram represents the genetic arrangement of the cluster responsible for penicillin synthesis in fungi such as *Penicillium* and *Aspergillus*, and highlights the relationship between the acvA and ipnA genes. The acvA gene, which is the longest, encodes the enzyme ACV synthetase, responsible for catalyzing the key first step in the biosynthetic pathway of penicillin. In contrast, the ipnA gene encodes isopenicillin N synthase, which is involved at a later stage of the process, while the aat gene participates in the final modifications necessary to obtain the active form of the antibiotic.

Both genes are separated by an intergenic region of approximately 872 base pairs, which plays an essential regulatory role, since it contains the promoters and shared transcriptional control elements. The arrows in the diagram indicate that the transcription of these genes occurs in opposite directions, which means that the transcription machinery moves in opposite directions from the same central region. This type of organization corresponds to a divergent transcription from a bidirectional promoter, allowing a coordinated and efficient expression of the genes involved in the production of penicillin and thus optimizing the use of genetic material.

In genomic analysis, the identification of sequences homologous to the UniRef90_A2QZ81 cluster supports the presence of the acvA gene (also known as pcbAB), responsible for encoding the ACV synthetase enzyme. This gene is a key element of the biosynthetic penicillin cluster described in fungi such as *Penicillium chrysogenum* and *Aspergillus nidulans* [5,13,32].

Like any gene, acvA is made up of a double helix of DNA formed by an ordered sequence of deoxyribonucleotides linked by phosphodiester bonds. Each nucleotide has a nitrogenous base (A, T, C, or G), a deoxyribose, and a phosphate group. The linear arrangement of these bases forms the primary structure of the gene and contains the information necessary to direct the synthesis of its corresponding protein [42].

In this case, the acvA gene encodes the enzyme δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS), a large nonribosomal peptide synthetase (NRPS) with more than 3,700 amino acid residues. This enzyme plays the initial and determining role in the biosynthetic pathway of penicillin by catalyzing the sequential binding of L-α-aminoadipate, L-cysteine and L-valine in an ATP-dependent reaction, forming the tripeptide ACV [23,26].

The LCA has a modular organization typical of NRPS, made up of the functional domains A (adenylation), C (condensation) and T (thiolation), each responsible for activating, binding and transporting the corresponding amino acids. In addition, it incorporates a thioesterase domain, whose function is to release the final product after the assembly of the tripeptide [17].

The ACV tripeptide, δ-(L-α-aminoadipil)-L-cysteinyl-D-valine, is the direct precursor of penicillin and other β-lactam antibiotics, such as cephalosporins and cephamycins (Elander, 2003). Each of the amino acids that make it up fulfills a specific function: L-α-aminoadipate constitutes the portion that will give rise to the characteristic side chain of the molecule; for its part, L-cysteine provides the sulfur atom essential for the subsequent formation of the thiazolidine ring. D-valine, unusual in its configuration in biological systems, plays a crucial role in the construction of the β-lactam ring, one of the most representative structures of penicillins [5].

The detection of the acvA gene marks the starting point of the biosynthetic pathway of β-lactam antibiotics. Its sequence encodes a multidomain enzyme responsible for assembling the ACV tripeptide, whose composition and molecular architecture are essential for the subsequent stages that lead to the formation of penicillin and other metabolites with therapeutic relevance [17,23].

Recalling that the complete genomic sequence of *A. salvadorensis* was generated by Macrogen (South Korea) in 2024, and in 2025 complementary analyses were carried out that allowed obtaining tables of enzymes, proteins and secondary metabolites. Among these results, entry UniRef90_A2QZ81 stands out, which presents the functional annotation *"the penicillin biosynthesis is catalysed by three enzymes encoded by acvA",* with a detection frequency of 31.7269 in the DNA sequence.

El identificador UniRef90_A2QZ81 corresponde a una proteína homóloga asociada al gen *acvA* (también denominado *pcbAB*), que codifica la enzima δ-(L-α-aminoadipil)-L-cisteinil-D-valina sintetasa (ACV sintetasa). Esta enzima multifuncional, perteneciente a la familia de las sintetasas de péptidos no ribosomales (NRPS), cataliza la primera reacción de la ruta biosintética de la penicilina, donde se forma el tripéptido ACV, precursor esencial de los antibióticos β-lactámicos.

> The detection of a protein homologous to the *acvA gene* in *A. salvadorensis* suggests that this fungus has the genetic potential to produce penicillin or a structural analogue, which represents a finding of high biotechnological relevance. The frequency of 31.7269 indicates a significant relative abundance of this sequence, reinforcing the evidence that the gene is present and possibly active.
>
> These results confirm that *Aspergillus salvadorensis* retains a biosynthetic cluster similar to that of classically penicillin-producing species, such as *P. chrysogenum*. However, cluster activity may depend on environmental and metabolic regulatory factors, so it is recommended to perform gene expression assays and secondary metabolite induction studies to verify their functionality. [16]

From a biotechnological perspective, the identification of the acvA *gene* and its associated counterparts with the penicillin biosynthesis pathway opens up new opportunities for research in the production of natural antibiotics or semi-synthetic derivatives, as well as for the evolutionary exploration of biosynthetic clusters in species of the genus *Aspergillus*.

Genetic evidence and potential for penicillin production. The coincidence with UniRef90_A2QZ81 is particularly relevant from a biotechnological perspective, since it is directly associated with the penicillin biosynthesis pathway. This group includes proteins encoded by the *ACVA gene*, which is responsible for the production of the enzyme ACV synthetase (*δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase*). This enzyme catalyzes the first step in the biosynthetic pathway of β-lactam antibiotics, by condensing three precursor amino acids – *L-α-aminoadipathe*, *L-cysteine* and *D-valine* – to form the tripeptide ACV, an essential precursor of penicillin. [10,23]

The detection of this homology in *Aspergillus salvadorensis* suggests that, like other species of the genus *Aspergillus*, this fungus possesses metabolic pathways specialized in the synthesis of secondary metabolites, including antibiotics, pigments, and other bioactive compounds. [39,41]

The genomic analysis that revealed the presence of the UniRef90_A2QZ81 protein sequence in the genome of *A. salvadorensis* constitutes solid evidence of the genetic potential of the organism to initiate the production of penicillin or an analogous β-lactam antibiotic, this highlights the importance for future therapeutic purposes.

Group UniRef90_A2QZ81 groups proteins with 90% or more sequence identity, indicating that the protein detected in *A. salvadorensis* has a high evolutionary and functional similarity to enzymes already characterized in other penicillin-producing fungi, such as *Penicillium chrysogenum* and *Aspergillus nidulans*. Consequently, the identified protein can be considered functionally equivalent to the known enzymatic machinery for penicillin biosynthesis.

The homologous protein detected is directly related to the *acvA* gene (also called *pcbAB),* which encodes the enzyme δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS). This multifunctional enzyme initiates the biosynthetic penicillin pathway, catalyzing the sequential condensation of three precursor amino acids to generate the ACV tripeptide, which constitutes the primary structural backbone of the antibiotic molecule. The presence of this sequence predicts, therefore, that *A. salvadorensis* possesses the genetic machinery essential for the initial synthesis of penicillin.

The genomic report of MACROGEN (South Korea, 2024) confirms that the biosynthetic pathway of β-lactam antibiotics is partially conserved in the genome of *Aspergillus salvadorensis*. This finding reinforces its potential as a model organism for bioprospecting for novel antimicrobial metabolites and suggests that it could produce natural penicillin or structurally related analogues under suitable culture conditions.

These results suggest that *A. salvadorensis* possesses the genetic machinery necessary for the biosynthesis of β-lactam compounds, which represents a significant finding in the search for new fungal sources of natural antibiotics [25]. In summary, genetic and functional evidence supports the biotechnological relevance of *A. salvadorensis* as an emerging source of bioactive compounds with potential pharmaceutical application already predicted above.

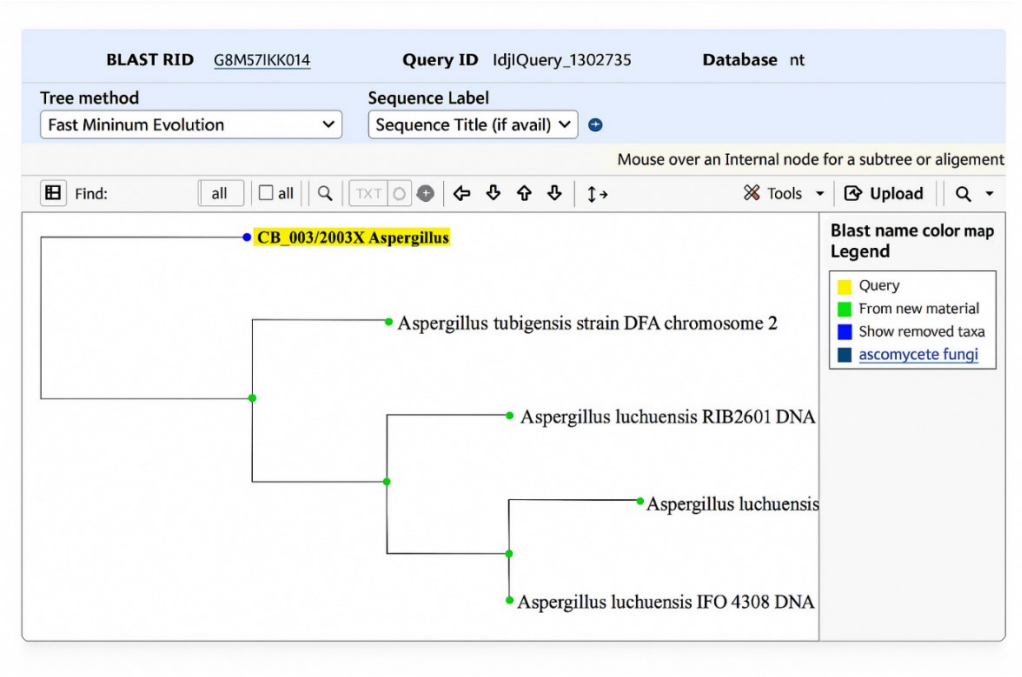


Fig. 2. Phylogenic tree representing *Aspergillus salvadorensis*.

In Fig 2. The phylogenetic analysis generated from the query sequence (>CB_001126055. *Aspergillus*) shows that the studied isolation is integrated within the clade corresponding to the genus *Aspergillus*, establishing a close evolutionary relationship with *Aspergillus luchuensis* and *Aspergillus tubingensis*. This clustering pattern places the sample within the general lineage associated with the Nigri section, a group widely recognized for its high genetic diversity, remarkable ecological plasticity, and importance in industrial applications [34,37].

Despite this evolutionary proximity, the genetic distance observed between the sequence analyzed and its most closely related species reveals a considerable level of divergence. This substantial difference constitutes solid evidence in favor of their taxonomic separation, supporting the proposal of *Aspergillus salvadorensis* sp. nov. as an independent species. The fact that the sequence is located in a well-supported and clearly differentiated clade confirms the existence of a unique lineage within the genus *Aspergillus*, consistent with previous analyses that integrate morphological, genomic and phylogenetic data [19].

From the biotechnological point of view, the phylogenetic position of *A. salvadorensis* acquires special relevance, since the members of the Nigri section are distinguished by their ability to produce a wide range of secondary metabolites with industrial applications, including hydrolytic enzymes, organic acids and bioactive compounds such as β-lactam antibiotics [14,15,20,30,38]. In this sense, its evolutionary location not only confirms its identity as a new species, but also suggests considerable biosynthetic potential. This makes *A. salvadorensis* a promising candidate for future research aimed at bioprospecting, the development of biotechnological processes and the exploration of fungal resources with pharmaceutical value in the Mesoamerican region.

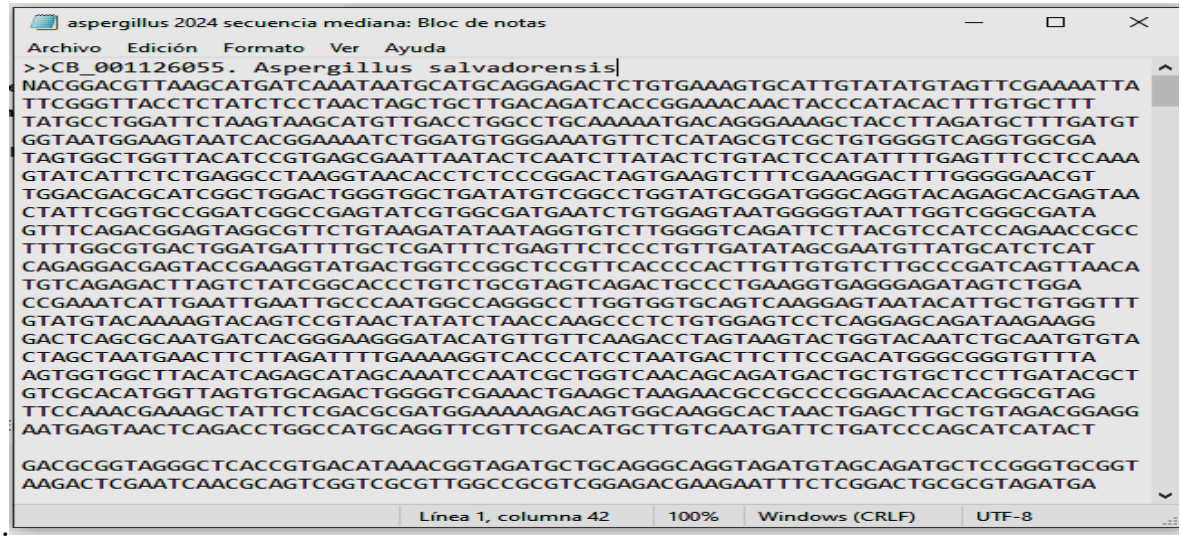


Table I. DNA *sequence Aspergillus salvadorensis*. Gen acvA .2024

Table 1. The analysis corresponding to the sequence with accession number CB_001126055 is shown, which is annotated within the genus *Aspergillus*. The bioinformatic processing of this sequence allowed the identification of the presence of the acvA gene, encoding the enzyme adenylyl peptide synthetase or ACV synthase (δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase). This enzyme plays an essential role in initiating the biosynthetic penicillin pathway, as it catalyzes the binding of the three precursor amino acids L-α-aminoadipate, L-cysteine and D-valine to form the tripeptide ACV, an immediate precursor molecule of the β-lactam nucleus characteristic of the antibiotic [26,33].

In species of the genus *Aspergillus* including *A. nidulans*, *A. oryzae* and *A. uessalvadorensis* the acvA gene is part of a conserved biosynthetic cluster associated with penicillin production. This cluster also includes the genes ipnA, which encodes isopenicillin N synthase responsible for the oxidative cyclization reaction, and aataA/penDE, whose enzymatic product acts as acyltransferase in the final stage of lateral modification of the molecule. The sequential and coordinated action of these genes ensures the progressive synthesis of the β-lactam metabolite, from the initial tripeptide to the structurally active form of the antibiotic. [3,10,23]

The analysis of conserved domains performed on the CB_001126055 sequence revealed the presence of the functional modules typical of *nonribosomal peptide synthetases* (NRPS). Among them, the following were identified: the adenylation domain A, responsible for selecting and activating precursor amino acids; the T or thiolation domain, which transfers substrates via a phosphopantheine residue; and the condensation C domain, responsible for catalyzing the sequential union of the amino acids that make up the ACV tripeptide. The estimated length of the acvA gene, between 11 and 15 kb, coincides with the sizes described for the equivalent NRPS in reference species of the genus *Aspergillus*, which supports its correct assignment as adenylyl-peptide synthetase. Overall, the results confirm that the CB_001126055 sequence, corresponding to an isolate of *Aspergillus* sp., does indeed contain the acvA gene, whose function is to catalyze the first step of the penicillin biosynthetic pathway by generating the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine. This finding reinforces the hypothesis that *Aspergillus salvadorensis* has the genetic and enzymatic potential necessary to initiate the synthesis of β-lactam compounds, positioning itself in the same

positionAs a biotechnological resource of interest in the search for new antibiotics or structural analogues. [10]

In the above sequence, identify the region encoding the **acvA gene** .The process would be as follows: **Identification of the correct reading frame**: We need to find the sequence that corresponds to the **acvA** gene, which may involve looking for specific signals, such as start codons (ATG) and end codons (TAA, TAG, TGA). **Translation of the DNA sequence to proteins**: Once the region encoding the **acvA** gene has been identified, we can translate it into the corresponding amino acid sequence. The **NCBI ORF Finder** program is used and gives us the following results based on the previous sequence:

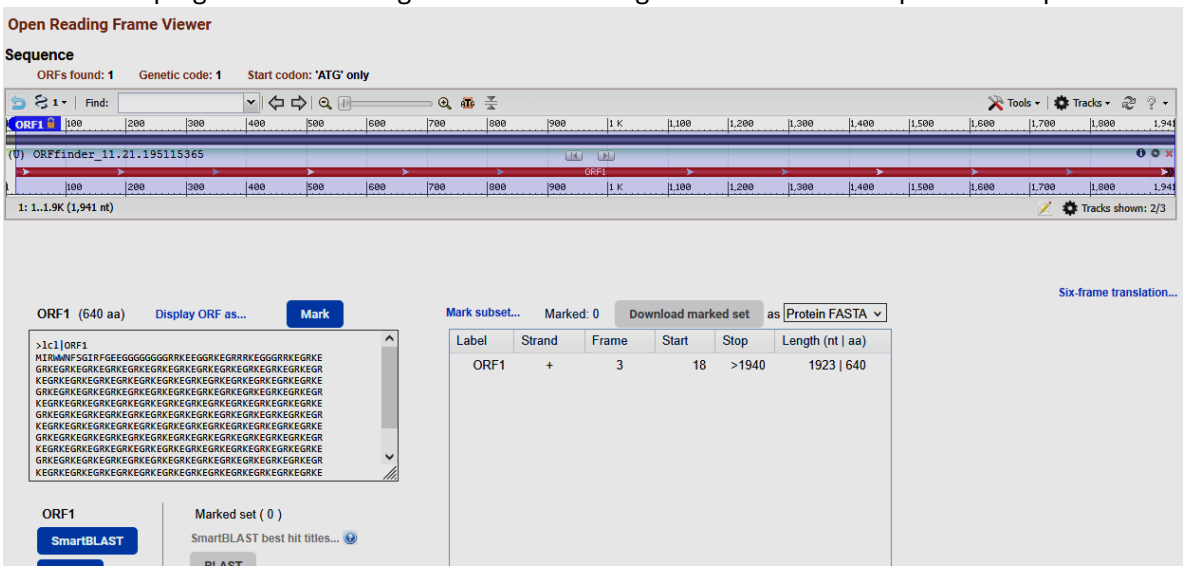


Fig 3. DNA sequence *Aspergillus salvadorensis*. Source: **NCBI ORF Finder.2025**

In Fig 3. Using a tool like **ORF Finder** you have identified an **open reading frame (ORF)** in your DNA sequence. This ORF is a region of the sequence that begins at an **ATG** start codon (which marks the beginning of translation into proteins) and ends at a stop codon (which signals the end of translation).In this case, the **ORF** identified in the sequence has a length of **640 amino acids**. The resulting amino acid sequence begins with the sequence "MIRNNSIFRGREKGKGGRGGGGGGGGGGG...", which represents the protein chain encoded by this ORF. The tool has found this ORF in the **DNA strand in a positive sense**, meaning that the sequence was read normally, from 5' to 3'. This ORF begins at position **18** of the DNA sequence and extends beyond position **1940**, covering a large portion of the sequence. The analysis also indicates that the ORF is in frame **3** of reading, meaning that the sequence has been read from the third nucleotide of the chain in the reading frame.

In general, fungi are eukaryotic organisms belonging to the kingdom *Fungi*, one of the five kingdoms traditionally used for the classification of living beings along with *Animalia*, *Plantae*, *Protista* and *Monera*. Unlike plants, they do not have chlorophyll or photosynthesis, so they depend on the absorption of organic matter from the environment to obtain energy and nutrients, the native species *salvadorensis* depends on the nacascol seed (Caeselpinio coriaria). They are distributed in practically all ecosystems on the planet and can manifest themselves both in unicellular forms such as yeasts, and in highly differentiated multicellular structures such as molds and fruiting bodies, with sizes ranging from microscopic organisms to visible macroscopic structures. Its classification is mainly

based on morphological and reproductive characteristics, which allows us to distinguish five main groups: Basidiomycota, Ascomycota, Glomeromycota, Zygomycota and Chytridiomycota. Basidiomycota are characterized by the formation of basidiocarps, where spores develop on basidia, while numerous Ascomycota are known for the production of secondary metabolites, including penicillin [27,44].

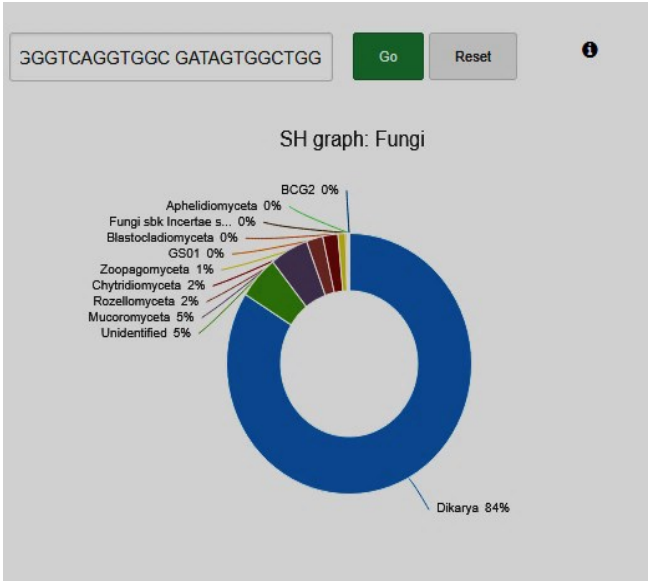
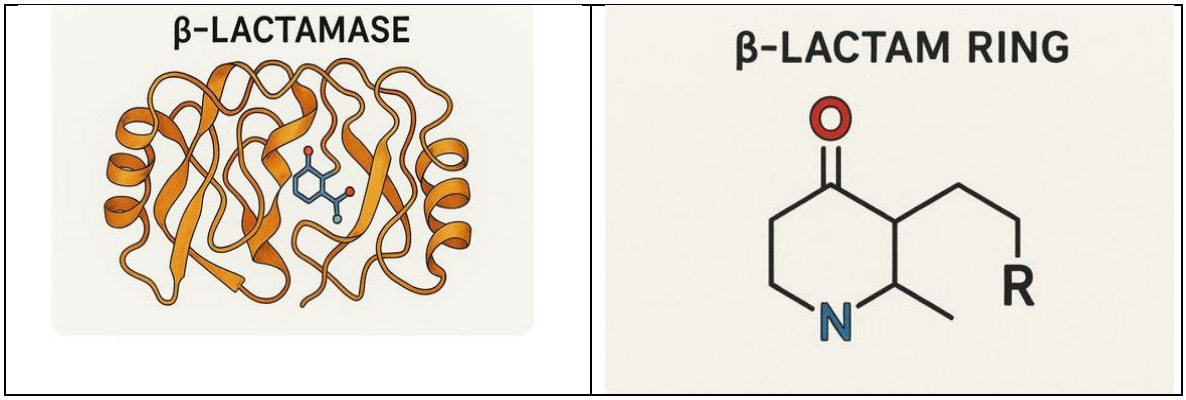


Fig 3. **Aspergillus salvadorensis *sequencing* . UNITE. 2025**

In Fig 3. A sequence of *A. salvadorensis* is shown, showing a graph of taxonomic distribution of fungi, based on data from genetic sequences. In the graph, it can be seen that the predominant group is the Dikarya (84%), which includes two large divisions of fungi: Ascomycota and Basidiomycota.

In the International Nomenclature Standard for Fungi of Madrid 2025 establishes that the priority prevails to the researcher who published first in an indexed journal, it does not matter if the sequence was later since it determines similarity of the bases with respect to other species, it does not give species, but the well-explained morphological characteristics together with innovative aspects determine a new species and then the sequencing is validated with deposit in Genbank plus validation name in Fungal Name or Mycobank.



Beta lactamase enzyme	Beta-lactam ring

Fig 4. Image of beta lactamase enzyme and beta-lactam ring. IA 2025

In Fig 4 The synthesis of penicillinase by *Aspergillus salvadorensis* leads to hydrolysis of the β-lactam ring present in antibiotics such as penicillins, which inactivates its antimicrobial activity. The enzymatic degradation of the β-lactam core gives the fungus an intrinsic resistance to these compounds and gives it a competitive advantage in ecological niches where antibiotic β-lactam residues exist. In this context, *A. salvadorensis* can establish and proliferate in microenvironments dominated by susceptible bacteria, displacing or limiting their growth. Although this species is not considered a relevant clinical pathogen, the potential capacity to produce penicillinase represents a biochemical defense mechanism that favors its persistence and adaptation in ecosystems exposed to antibiotic pressure in an oxidative stress.

If *Aspergillus salvadorensis* produces penicillinase in a laboratory environment, this enzyme exerts a direct effect on the microbial dynamics of the culture. The penicillinase released into the extracellular medium breaks the β-lactam ring of the penicillins incorporated into the culture media, thereby eliminating their inhibitory activity against susceptible bacteria. As a consequence, microorganisms that would normally be suppressed by the presence of the antibiotic can continue to proliferate due to the neutralization of the compound. This behavior is clearly observed in cultures grown on Mueller Hinton agar, where the action of the penicillinase generates areas of bacterial growth within zones that, in theory, should display inhibition.

Likewise, the degradation of the antibiotic by the fungus alters the selectivity of the medium. An agar medium supplemented with penicillin loses its ability to favor or isolate resistant bacteria, since the fungal enzyme reduces the effective concentration of the antimicrobial to subinhibitory levels. This leads to misleading results in antibiotic susceptibility testing, as bacteria classified as susceptible may appear resistant or show normal growth when they coexist with the fungus.

In agar diffusion assays, the presence of *Aspergillus salvadorensis* can produce protective zones around its colonies, similar to a “shield effect,” where sensitive bacteria encounter a microenvironment devoid of, or with very low levels of, penicillin due to the localized action of penicillinase. Similarly, in liquid cultures, the enzymatic activity can rapidly break down the antibiotic, altering bacterial growth curves and affecting the reproducibility of microbiological assays.

In summary, the production of penicillinase makes *Aspergillus salvadorensis* an organism capable of interfering with antimicrobial susceptibility tests, selective media, and microbial interaction experiments. Its ability to inactivate penicillins significantly modifies the behavior of susceptible bacterial populations, allowing their survival, proliferation, and expansion under conditions that would normally inhibit them.

Discussion

Currently, the study of fungi with medicinal applications is going through a period of extensive growth, driven by the interest in discovering new species with therapeutic potential and in elucidating the mechanisms by which these organisms influence the modulation of the human immune system. Recent advances in molecular biology, genomics, and biotechnology have allowed the targeted manipulation of various fungal strains, facilitating the optimization of the synthesis and isolation of bioactive metabolites with potential pharmacological use [24,15,41]).

Within this context, species of the genus *Penicillium* are commonly recognized as frequent contaminants of multiple substrates and are characterized by their ability to produce different mycotoxins, including ochratoxin A (OTA) and cyclopiazonic acid (CPA) [29]. These filamentous fungi are distinguished by forming colonies of generally rapid growth, composed of dense aggregations of conidiophores and which, in most cases, have greenish hues. The colonial texture is an important diagnostic feature, and can be velvety, woolly, funiculous or fasciculated, varying according to the organization of the conidiophores. [14,15,16,21]

The asexual reproductive structure characteristic of the genus is the conidiophore, organized in a branched architecture similar to a "brush" or penicillium. This structure can have different degrees of branching and complexity, which is essential for the correct taxonomic identification of the group [22].

Fungi are made up of cells that, in most cases, are organized into filaments called hyphae. These structures usually have transverse septa called septa, which is why they are known as septate hyphae. However, certain fungal groups develop hyphae without septa, called aseptate or coenocytic hyphae. In fruiting bodies, some hyphae acquire a high degree of functional specialization: while some play a structural role, such as skeletal or enveloping hyphae, others act as conduits for the internal transport of nutrients, configuring a system similar to a conductive tissue. The set of these hyphae makes up the mycelium, which constitutes the vegetative phase of the organism. Most fungi also have the ability to generate asexual reproductive structures; thus, the Ascomycota produce ascomas and the Basidiomycota form basidioms, equivalent to the mushrooms found in natural environments and functionally comparable to the fruits of a tree. Generally speaking, fungal architecture includes a subterranean vegetative phase (mycelium) and a visible asexual phase (fruiting bodies) [27]. The study fungus *A. salvadorensis* also belongs to this group.

The genus *Penicillium*, widely distributed in various ecosystems, comprises about 350 recognized species. This group is particularly relevant due to its industrial importance and its ability to synthesize mycotoxins with adverse effects on mammals and other animals. Such secondary metabolites can occur both in agricultural crops and during the storage of raw materials. Among them, Ochratoxin A (OTA) stands out, considered of special importance for its renal toxicity and for its carcinogenic, teratogenic and immunosuppressive potential. Originally discovered in South Africa in 1965, this mycotoxin is mainly associated with *Penicillium verrucosum* and *Penicillium nordicum*, species recognized as the main producers of OTA within the genus [1,7,36].

These species proliferate more frequently in regions with a temperate to cold climate and usually develop in cereals stored under unsuitable humidity and temperature conditions, which favors the synthesis of mycotoxins such as OTA (Bionte, 2025). A . *salvadorensis* grows Latitude: 13.8,Longitude: -88.1,UFI: -1147125,UNI: -1691519,UTM: CA82,JOG: ND16-10 which allows it to adapt to this climate.

The genus *Penicillium*, ascribed to the phylum Ascomycota, was first described by the German mycologist Johann Heinrich Friedrich Link in 1809 (Link, 1809). In its beginnings it was located within the Deuteromycetes or "imperfect fungi", since only its asexual or anamorphic phase was known. With the advance of mycology, it was determined that several species actually corresponded to sexual states belonging to the genera *Eupenicillium* and *Talaromyces*, both included within the ascomycetes. Hyphae of the anamorphous phase are typically hyaline and septate, and in culture the colonies show shades ranging from blue-green to pink. Its filamentous growth and saprophytic nature explain its frequent appearance as molds on a wide variety of organic substrates, reflecting its remarkable ecological plasticity [11,37]. The Salvadorensis species is part of this group Ascomycota.

Many species of the genus *Penicillium* are of considerable importance for human activities. The production of the antibiotic penicillin, the first massively used antimicrobial drug, was obtained from *Penicillium chrysogenum*. [13]Similarly, the texture, characteristic aroma and streak formation in blue cheeses are due to the lipolytic and proteolytic activity of species of the genus, capable of hydrolyzing lipids and proteins during the maturation processes. Their efficiency as saprophytes derives from the abundant secretion of hydrolytic enzymes, which allows them to rapidly degrade organic matter. In addition, *Penicillium* species can colonize numerous types of substrates and their spores are common indoor air pollutants. Some species act as pathogens in agricultural crops, both in the field and during post-harvest storage, and many strains produce mycotoxins with adverse effects on human health. In contrast, other species are favorably used in fermentation processes and in the synthesis of antibiotics. Their distribution is cosmopolitan and they have the ability to develop in extreme environments of temperature, salinity, pH or water availability. Asexual reproduction can occur through fragmentation of mycelium, formation of sclerotia resistance structures formed by thick-walled mycelium, or through the production of conidia from conidiophores, where phylalides generate conidia after successive nuclear divisions [16,19,29].

Species belonging to the genus *Aspergillus* are distinguished by their remarkable ability to synthesize a wide range of secondary metabolites, including mycotoxins, pigments, alkaloids and other bioactive molecules that contribute to their adaptation, survival and defense against various environmental stressors. The existence of specialized metabolic pathways for the production of these compounds reflects the intrinsic potential of the fungus to generate substances with biological relevance. In this sense, recent studies have indicated that a species locally identified in El Salvador, called *Aspergillus salvadorensis*, has the ability to produce black pigments of natural origin. At the same time, research focused on fungi with medicinal properties continues to expand, encompassing the identification of new species with potential therapeutic applications, the analysis of their mechanisms of interaction with the human immune system, and the use of advanced molecular biology tools for the genetic optimization of fungal strains aimed at increasing the production of bioactive compounds [18,39].

The production of penicillin in research settings is a carefully regulated biotechnological process that comprises three general phases: fermentation, extraction and purification [10]. This scheme, implemented in stirred flasks or small-scale bioreactors, aims to reproduce in a controlled manner the conditions used in the industrial production of the antibiotic.

During the fermentation stage, the main purpose is to promote the formation of penicillin, a secondary metabolite produced by certain fungi. For this purpose, strains of *Aspergillus nidulans* or, more frequently, *Penicillium chrysogenum*, historically recognized as the fundamental microorganism in obtaining this antibiotic, are usually used [13,5]). The fungus develops in a nutritious liquid medium that incorporates appropriate sources of carbon – such as lactose or combinations of this with other

sugars and complex sources of nitrogen derived from plant materials or microbial extracts, the nacascol seed precisely contains these nutrients, which makes it exclusive as a source of carbon because the seed has carbohydrates, proteins as a source of hydrogen for energy and growth, lipids as energy. [39.40]

When a particular type of penicillin, such as penicillin G, is required, a compound that acts as a precursor of the side chain is added to the culture; in this case, phenylacetic acid directs the metabolic pathway towards the generation of the corresponding antibiotic [9,10].

The culture begins with a high density of spores and is maintained under controlled conditions of agitation, temperature and pH for several days, so that both the growth of the microorganism and the accumulation of the antibiotic in the medium are optimized [10,8].

The second phase, corresponding to extraction, begins once fermentation is complete. At this stage, penicillin is dispersed in the liquid medium, so it is necessary to separate the fungal biomass by means of clarification techniques that allow obtaining a solids-free filtrate. Subsequently, physicochemical procedures are carried out that modify the ionization state of the antibiotic and facilitate its transfer between aqueous and organic phases, which makes it possible to recover and enrich it [26]. These separation cycles can be repeated in order to increase the purity level of the resulting compound [5,10].

The third phase, known as purification and crystallization, involves the treatment of the penicillin present in the aqueous solution using separation techniques such as precipitation or chromatography procedures in order to remove remaining impurities. Subsequently, the antibiotic undergoes a crystallization process, usually in the form of its sodium or potassium salts, applying controlled conditions that regulate physicochemical parameters of the system. In this way, a crystalline penicillin of high purity is obtained, suitable for subsequent analysis, formulation or pharmaceutical application [10,35].

Conclusions

The acvA gene in MACROGEN INC in the DNA chain of *Aspergillus salvadorensis was reported by MACROGEN INC SOUTH KOREA*, so it is concluded that it is a precursor of the synthesis of Penicillin, as stated in the archives of the microbiology department of the Faculty of Medicine. In genomic analysis, the identification of sequences homologous to the UniRef90_A2QZ81 cluster supports the presence of the acvA gene (also known as pcbAB), responsible for encoding the ACV synthetase enzyme. This gene is a key element of the penicillin biosynthetic cluster.

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Conflicts of Interest. The author declares no conflicts of interest.

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