The Role of Gene X Deletion in Enhancing Secondary Metabolite Production in Fungi

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CERTIFICATE

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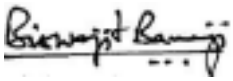
We wish her every success in all her endeavors.



Human Resources Department
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Declaration of Originality

I, Mansi Singh, Roll Number 602204012, hereby declare that the thesis titled “The Role of Gene X Deletion in Enhancing Secondary Metabolite Production in Fungi,” submitted in fulfillment of the requirements for the degree of Master of Technology (M.Tech) in Biotechnology, is the result of my original research conducted under the supervision of Prof. Manoj Baranwal.

I affirm that the content of this thesis is my own work and has not been submitted, either in whole or in part, to any other academic institution or university for the award of any degree or diploma.

I also certify that I have appropriately cited all sources of information and that this work is free from any form of plagiarism.

Mansi Singh

Supervisor Certificate

This is to certify that the thesis entitled "The Role of Gene X Deletion in Enhancing Secondary Metabolite Production in Fungi" submitted by Mansi Singh (Roll Number: 602204012) in fulfillment of the requirements for the award of the degree of Master of Technology (M.Tech) in Biotechnology is a record of original research work carried out by her under my supervision and guidance.

The results presented in this thesis have not been submitted in whole or in part to any other institute or university for the award of any degree or diploma.

I wish her all the best in her future endeavors.

Prof. Manoj Baranwal
Professor

Department of Biotechnology
Thapar Institute of Engineering and Technology

Dedication

I would like to express my deepest gratitude to Dr. Arvind Kumar Verma, my manager at Biocon Ltd., for his invaluable support and guidance throughout this journey. His encouragement and insights have been instrumental in the completion of this thesis.

I am also profoundly thankful to Prof. Manoj Baranwal, my guide at Thapar University, for his continuous mentorship and expert advice. His guidance has been a cornerstone of my research, and I am deeply appreciative of his contributions.

This thesis would not have been possible without their unwavering support and belief in my work.

Mansi Singh

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Table of content

Abstract

The production of secondary metabolites in fungi is tightly regulated by various genetic and environmental factors. One of the key regulatory mechanisms is carbon catabolite repression (CCR), mediated by the transcriptional repressor Gene X. This thesis explores the impact of gene X gene deletion on secondary metabolite production in several fungal species. Additionally, the thesis discusses the use of In-Fusion cloning as a technique for constructing gene deletion cassettes, facilitating targeted genetic modifications in fungi. The study combines literature review, experimental data, and comprehensive analysis to provide a detailed understanding of the potential applications of gene X deletion and In-Fusion cloning in fungal biotechnology.

1. Introduction

Fungi are prolific producers of secondary metabolites, which have diverse applications in medicine, agriculture, and industry. These metabolites include antibiotics, mycotoxins, and other bioactive compounds. The regulation of secondary metabolism in fungi is complex, involving numerous genetic and environmental factors. One of the central regulatory mechanisms is carbon catabolite repression (CCR), which ensures that fungi utilize the most favorable carbon source available. The transcriptional repressor Gene X plays a crucial role in mediating CCR by binding to specific sequences in the promoters of genes involved in secondary metabolism, thereby repressing their expression.

Recent studies have demonstrated that the deletion of the gene X gene can lead to a significant increase in secondary metabolite production in various fungal species. This has opened up new avenues for enhancing the yield of valuable metabolites through genetic engineering. In this context, the development of efficient methods for constructing gene deletion cassettes is essential. In-Fusion cloning is a powerful technique that enables the seamless assembly of DNA fragments without the need for restriction enzymes, making it particularly suitable for constructing gene deletion cassettes.

This thesis aims to provide a comprehensive analysis of the impact of gene X gene deletion on secondary metabolite production in fungi and to explore the potential of In-Fusion cloning in fungal biotechnology. By integrating literature review, experimental data, and detailed analysis, this study seeks to contribute to the understanding of fungal metabolism and to advance the methods used in fungal genetic engineering.

2. Literature Review

2.1 Carbon Catabolite Repression in Fungi

Carbon catabolite repression (CCR) is a regulatory mechanism that allows fungi to preferentially utilize the most favorable carbon source available in the environment. This mechanism ensures that the metabolic machinery is optimally utilized, conserving energy and resources. CCR is mediated by several transcriptional regulators, with Gene X being one of the most extensively studied in filamentous fungi. [1]

Gene X represses the transcription of genes involved in the metabolism of secondary carbon sources when a preferred carbon source, such as glucose, is present.[1] This repression is achieved through the binding of Gene X to specific DNA sequences in the promoters of target genes, thereby blocking their transcription.[2]

2.2 Role of Gene X in Fungal Metabolism

Gene X is a zinc-finger transcription factor that binds to consensus sequences known as carbon catabolite repression elements (CCREs) in the promoters of target genes. The binding of Gene X to these sequences prevents the transcription of genes involved in the utilization of secondary carbon sources and the biosynthesis of secondary metabolites.[3]

In the absence of glucose or other preferred carbon sources, Gene X is inactivated, allowing the expression of genes required for the metabolism of alternative carbon sources. This regulatory mechanism ensures that fungi efficiently switch between different metabolic pathways in response to changes in environmental carbon availability.[4]

2.3 Impact of gene X Deletion on Secondary Metabolite Production

The deletion of the gene X gene has been shown to significantly enhance the production of secondary metabolites in various fungal species. For example, in Aspergillus flavus, the deletion of gene X leads to the derepression of aflatoxin biosynthetic genes, resulting in increased aflatoxin production even when grown on less favorable carbon sources like maltose.[1],[5]

Similarly, in *Penicillium chrysogenum*, the deletion of gene X results in increased expression of penicillin biosynthetic genes, leading to higher penicillin yields.[2]. In *Acremonium chrysogenum*, the deletion of gene X enhances the production of cephalosporin C by relieving the repression of key biosynthetic genes.[5],[6]

These findings highlight the potential of gene X deletion as a strategy for enhancing the production of valuable secondary metabolites in fungi. However, the effects of gene X deletion can vary depending on the species and the specific metabolic pathways involved.[4]

2.4 In-Fusion Cloning: Principles and Applications

In-Fusion cloning is a versatile and efficient molecular cloning technique that allows for the seamless assembly of DNA fragments without the need for restriction enzymes. This method relies on the incorporation of short homologous sequences (15-20 base pairs) at the ends of the DNA fragments to be joined. These homologous sequences facilitate the annealing of the fragments, enabling their insertion into a linearized vector in a single step.[7],[8]

The In-Fusion cloning process involves the preparation of the insert DNA fragments and the linearization of the vector. The insert fragments are amplified using PCR primers that incorporate the necessary homology sequences at their ends.[8] The linearized vector and the PCR-amplified insert fragments are then combined with the In-Fusion HD enzyme mix and incubated at 50°C, allowing the exonuclease activity of the enzyme to generate single-stranded overhangs that anneal to the complementary sequences on the neighboring fragments. [7],[9]

In-Fusion cloning offers several advantages, including high efficiency, the ability to clone large inserts (up to 15 kb), and the capacity to assemble multiple fragments in a single reaction. This technique is particularly useful for constructing gene deletion cassettes for fungi, facilitating targeted genetic modifications.[7]

2.5 Creation of deletion cassette for the gene X gene using In-Fusion cloning

To create a deletion cassette for the gene X gene using In-Fusion cloning, the following steps can be followed:

1. Design primers: Design primers to amplify the 5' and 3' flanking regions of the gene X gene, incorporating the necessary homology sequences for In-Fusion cloning. The forward primer for the 5' flank should include 15-20 base pairs of homology to the vector's 5' end, while the reverse primer should have 15-20 base pairs of homology to the 5' end of the selectable marker (e.g., hygromycin resistance gene, hph).[8] /10/Similarly, the forward primer for the 3' flank should have 15-20 base pairs of homology to the 3' end of the selectable marker, and the reverse primer should include 15-20 base pairs of homology to the vector's 3' end.[11]
2. Amplify flanking regions: Use PCR to amplify the 5' and 3' flanking regions of the gene X gene using the designed primers. The selectable marker (e.g., hph) can be amplified from a template plasmid or obtained as a linear fragment.[11]
3. Linearize the vector: Linearize the desired vector (e.g., pBluescript) using a suitable restriction enzyme or by inverse PCR. The linearized vector should have compatible ends for the In-Fusion reaction.[9]
4. Perform In-Fusion reaction: Mix the linearized vector, the amplified 5' flank, the selectable marker, and the amplified 3' flank in an In-Fusion reaction. Incubate the

mixture at 50°C for 15 minutes to allow the exonuclease activity of the In-Fusion enzyme to generate single-stranded overhangs and facilitate the annealing of the fragments.[5]/9/

5. Transform and select: Transform the In-Fusion reaction mixture into competent E. coli cells and select for transformants on appropriate antibiotic-containing media. Screen the resulting colonies for the presence of the assembled deletion cassette using colony PCR or restriction enzyme digestion.[11]
6. Verify the deletion cassette: Confirm the integrity of the assembled deletion cassette by sequencing the insert region. The deletion cassette should contain the 5' flank, the selectable marker, and the 3' flank in the correct orientation.[11]

The resulting deletion cassette can then be used for fungal transformation to generate gene X deletion mutants. The selectable marker allows for the identification of transformants, while the flanking regions facilitate the targeted integration of the cassette into the fungal genome via homologous recombination.

In-Fusion cloning offers several advantages for the construction of gene deletion cassettes, including its efficiency, flexibility, and the ability to assemble multiple fragments in a single reaction.[4],[5] This method has been successfully applied to various fungal species, including *Neurospora crassa* and *Aspergillus nidulans*, for high-throughput gene deletion projects.[11]

3. Objective

This study is designed with the following objective:

To develop a Gene X deletion cassette to evaluate the impact of Gene X deletion on secondary metabolite production

To develop a Gene X deletion cassette using molecular biology techniques such as PCR, In-Fusion cloning, and transformation; evaluate the impact of Gene X deletion on secondary metabolite production through shake flask trials and HPLC analysis; and elucidate Gene X's regulatory role in secondary metabolism via comprehensive molecular and biochemical analyses, including gene expression profiling and statistical validation.

4. Materials and Methods

Molecular Biology Techniques

The methodological framework employed in this research endeavor encompasses an intricate series of molecular biology techniques, each meticulously orchestrated to contribute to the

overall success of the project. This section delineates the procedures and principles underlying the core techniques used: Polymerase Chain Reaction (PCR), Cloning, and Transformation.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a cornerstone technique in molecular biology, pivotal for the amplification of specific DNA sequences. This section details the components and steps involved in the PCR process.

Components of PCR

1. **DNA Template:** The segment of DNA intended for amplification. The quality and purity of the DNA template are crucial, as contaminants can inhibit the PCR process.
2. **Primers:** Short, single-stranded DNA sequences, typically 18-30 nucleotides long, designed to be complementary to the flanking regions of the target DNA sequence. The primers provide starting points for DNA synthesis by the DNA polymerase.
3. **DNA Polymerase:** An enzyme that synthesizes new DNA strands by adding nucleotides to the primers. Taq polymerase, derived from the thermophilic bacterium *Thermus aquaticus*, is commonly used due to its stability at high temperatures.
4. **Nucleotide Mix:** A mixture of the four deoxyribonucleotides (dATP, dTTP, dCTP, dGTP) necessary for the construction of new DNA strands. The concentration of each nucleotide is typically 200 μM.
5. **Buffer Solution:** A solution that provides the optimal chemical environment for the DNA polymerase to function efficiently. The buffer usually contains Tris-HCl, KCl, and MgCl2, with pH adjusted to 8.3-8.8 at 25°C.

PCR Steps

1. **Denaturation:** The reaction mixture is heated to 94-98°C for 20-30 seconds to separate the double-stranded DNA into single strands. This high temperature breaks the hydrogen bonds between the complementary bases. (Figure 1)
2. **Annealing:** The temperature is lowered to 50-65°C for 20-40 seconds, allowing the primers to bind (anneal) to their complementary sequences on the single-stranded DNA. The exact annealing temperature depends on the melting temperature (Tm) of the primers, which is influenced by their length and GC content. (Figure 1)
3. **Extension:** The temperature is raised to 72-75°C for 30-60 seconds, enabling the DNA polymerase to synthesize new DNA strands by adding nucleotides to the primers. The extension time depends on the length of the target DNA, with a typical rate of 1 kb per minute for Taq polymerase. (Figure 1)
4. **Repeat:** The cycle of denaturation, annealing, and extension is repeated 20-40 times, resulting in the exponential amplification of the target DNA sequence. Each cycle

doubles the number of DNA molecules, leading to a geometric increase in the amount of target DNA. (Figure 1)

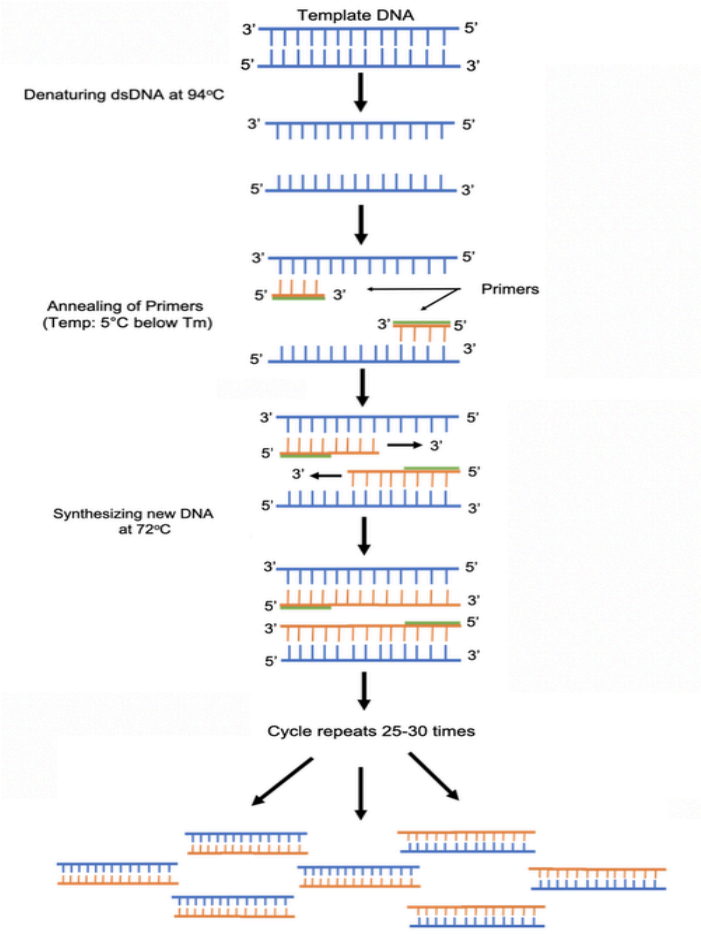

Figure 1: Polymerase Chain Reaction[1]

PCR is an indispensable tool in cloning, genetic testing, and DNA sequencing, enabling rapid and efficient amplification of specific DNA sequences.

Cloning

Molecular cloning is a fundamental technique in genetic engineering, biotechnology, and recombinant DNA technology. It involves the creation of recombinant DNA molecules and their propagation in host cells. The following steps outline the cloning process.

[1] *Addgene - What is Polymerase Chain Reaction, (n.d) https://addgene.org/protocols/pcr/*

Molecular Cloning Steps

1. **Isolation of DNA:** The target DNA (e.g., a gene) is isolated from the source organism using techniques such as cell lysis, centrifugation, and purification. Methods such as phenol-chloroform extraction and ethanol precipitation or commercial DNA extraction kits are commonly used to purify the DNA.
2. **Vector Selection:** A suitable vector (plasmid or viral genome) is chosen to carry the target DNA. The vector must have an origin of replication, a selectable marker (e.g., antibiotic resistance gene), and unique restriction enzyme sites for cloning.
3. **Digestion:** Both the target DNA and the vector are cut with the same restriction enzymes to create compatible sticky ends. Restriction enzymes recognize specific palindromic DNA sequences and cleave the DNA at or near these sites. The digestion reaction typically involves incubating the DNA with the enzyme at the recommended temperature (usually 37°C) for 1-2 hours. (Figure 2)
4. **Ligation:** The target DNA and vector are mixed together and joined by DNA ligase, forming recombinant DNA molecules. The ligation reaction involves the formation of phosphodiester bonds between the 3'-hydroxyl and 5'-phosphate ends of the DNA fragments, facilitated by the enzyme T4 DNA ligase in the presence of ATP. (Figure 2)
5. **Transformation:** The recombinant DNA is introduced into host cells (usually bacteria) through a process called transformation. Methods for making cells competent include chemical treatment with calcium chloride and electroporation, which uses an electrical field to increase cell membrane permeability.
6. **Screening:** Selective markers (e.g., antibiotic resistance genes) are used to identify cells that have taken up the recombinant DNA. Screening methods may also include blue-white screening, where the presence of a functional lacZ gene allows for the identification of colonies with recombinant plasmids.
7. **Culturing and Selection:** Transformed cells are cultured on selective media containing antibiotics or other selective agents. Only cells that have successfully taken up the recombinant DNA will grow and form colonies on the selective media.

Cloning is pivotal for manipulating and replicating specific genes or DNA fragments, thereby enabling advancements in genetic engineering and biotechnology.

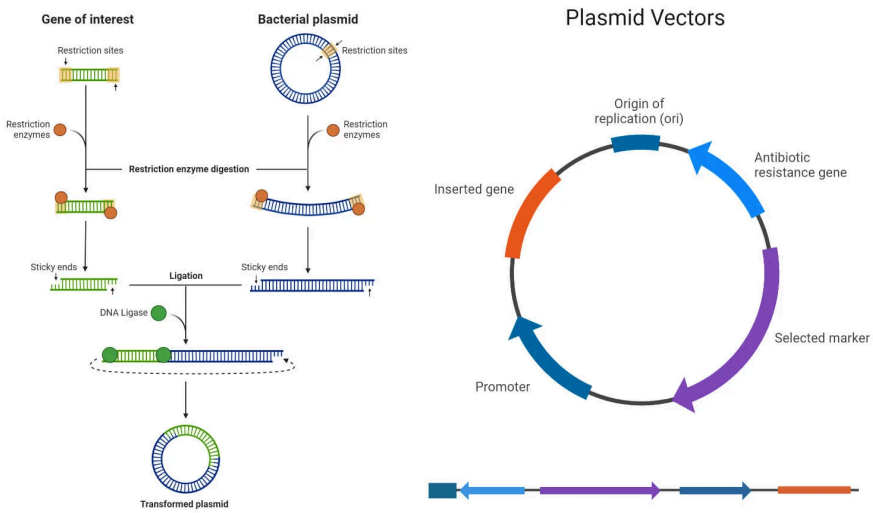

Figure 2: DNA Cloning: Steps[2]

Transformation

Transformation is a technique used to introduce foreign DNA into host cells, facilitating the expression of desired traits or proteins. The following steps describe the transformation process.

Transformation Steps

1. **Preparation of Host Cells:** Host cells (e.g., bacteria) are made competent for transformation through chemical treatment (e.g., calcium chloride) or electroporation. Chemical treatment involves incubating the cells with a solution of calcium chloride, which increases the permeability of the cell membrane. Electroporation involves subjecting the cells to a brief electrical pulse, creating temporary pores in the cell membrane through which DNA can enter.
2. **Mixing DNA and Host Cells:** The prepared host cells are combined with the foreign DNA (plasmid or other DNA fragments). This mixture is typically incubated on ice for a short period to allow the DNA to bind to the cell surface.

[2] *Tamang (n.d.), DNA Cloning: Principle, Steps, Components, Methods, Uses. Microwave Notes. https://microbenotes.com/dna-cloning/*

3. **Heat Shock:** The cell-DNA mixture is briefly exposed to a temperature change (heat shock) by placing the mixture at 42°C for 30-60 seconds, followed by rapid cooling on ice. This heat shock step facilitates the uptake of DNA by the host cells. (Figure 3)
4. **Recovery:** Transformed cells are allowed to recover in a nutrient-rich medium (e.g., LB broth) at 37°C for 30-60 minutes. During this recovery period, the cells express the antibiotic resistance gene and repair any damage to their membranes. (Figure 3)
5. **Selection:** Selective markers (e.g., antibiotic resistance genes) are used to identify cells that have successfully taken up the foreign DNA. The transformation mixture is plated on agar plates containing the appropriate antibiotic. Only cells that have incorporated the plasmid DNA will grow and form colonies on the selective plates. (Figure 3)

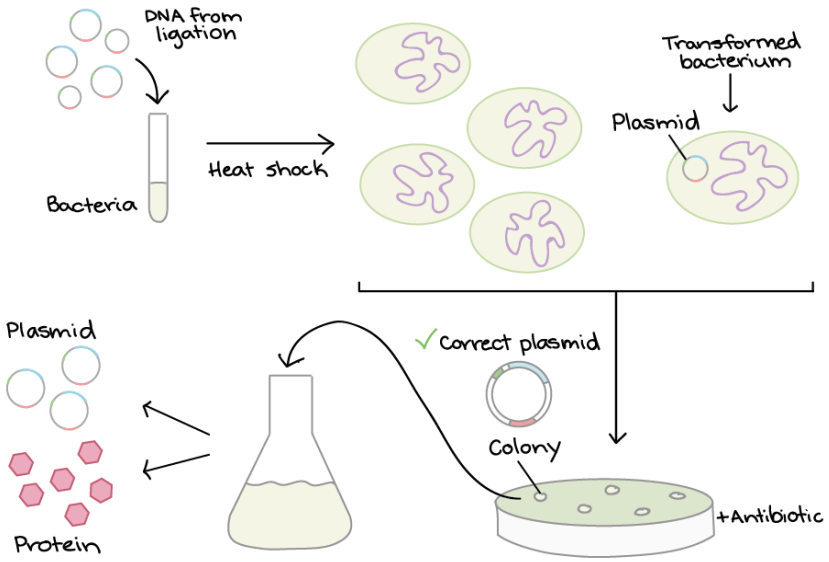

Figure 3: Bacterial Transformation and Selection [3]

Transformation is essential in genetic engineering and biotechnology, as it enables the introduction of specific genes or DNA constructs into host cells, leading to the expression of desired traits or proteins. This technique is crucial for the production of recombinant proteins, the study of gene function, and the development of genetically modified organisms (GMOs).

[3] *Khan Academy, Overview: DNA cloning, (n.d), https://www.khanacademy.org/science/ap-biology/gene-expression-and-regulation/biotechnology/a/overview-dna-cloning*

Agrobacterium-Mediated Transformation in Fungi

Agrobacterium-mediated transformation is a widely used technique for introducing foreign DNA into fungal genomes. This method leverages the natural ability of *Agrobacterium tumefaciens* to transfer DNA into host cells. Here, we detail the transformation process for the fungi *Coleophoma empetri* and *Aspergillus nidulans*.

Materials and Methods

Strains and Plasmids

1. **Agrobacterium Strain:** *Agrobacterium tumefaciens* strain (e.g., AGL-1) containing the binary vector with the gene of interest.
2. **Fungal Strains:** *Coleophoma empetri* and *Aspergillus nidulans*.
3. **Binary Vector:** Plasmid with the gene of interest, selectable marker (e.g., hygromycin B resistance gene), and necessary T-DNA borders for transfer.

Preparation of Agrobacterium tumefaciens

1. **Culture Initiation:**
    a. Inoculate a single colony of *A. tumefaciens* into 5 mL of LB medium containing appropriate antibiotics (e.g., rifampicin, kanamycin).
    b. Incubate overnight at 28°C with shaking at 200 rpm.
2. **Subculture:**
    a. Transfer 1 mL of the overnight culture into 50 mL of LB medium with antibiotics.
    b. Incubate at 28°C with shaking until the OD600 reaches 0.5-0.6 (approximately 4-6 hours).
3. **Induction:**
    a. Centrifuge the culture at 4,000 x g for 10 minutes and resuspend the pellet in 50 mL of induction medium (IM) containing 100 µM acetosyringone.
    b. Incubate at 28°C with shaking at 200 rpm for 4-6 hours. (Figure 4)

Preparation of Fungal Spores

1. **Spore Harvesting:**
    a. Grow *C. empetri* and *A. nidulans* on PDA plates at 25°C and 37°C, respectively, until sporulation (approximately 7-10 days).
    b. Harvest spores by adding sterile water to the plate and gently scraping the surface with a sterile loop.

2. **Spore Suspension:**

    a. Filter the spore suspension through sterile cheesecloth to remove mycelial fragments.
    b. Adjust the spore concentration to 1 x 10^6 spores/mL in sterile water. (Figure 4)

Co-cultivation

1. **Mixing:** Combine 1 mL of the induced *A. tumefaciens* suspension with 1 mL of the fungal spore suspension in a sterile Eppendorf tube.
2. **Co-cultivation on Filters:**
    a. Place a sterile filter paper (e.g., Whatman) on an IM agar plate.
    b. Pipette 100 µL of the *Agrobacterium*-spore mixture onto the filter paper.
    c. Incubate the plate at 25°C for *C. empetri* and 28°C for *A. nidulans* for 2-3 days in the dark. (Figure 4)

Selection of Transformants

1. **Transfer to Selection Medium:**
    a. After co-cultivation, transfer the filter paper to a selection medium (SM) plate containing the appropriate antifungal (e.g., hygromycin B) and cefotaxime to kill *Agrobacterium*. (Figure 4)
    b. Incubate at 25°C for *C. empetri* and 37°C for *A. nidulans* until transformants appear (5-10 days).
2. **Isolation of Transformants:**
    a. Pick individual colonies and transfer to fresh SM plates.
    b. Incubate at the appropriate temperature until colonies grow.

Molecular Confirmation

1. **Genomic DNA Extraction:**
    a. Extract genomic DNA from putative transformants using a fungal DNA extraction kit or CTAB method.
2. **PCR Confirmation:**
    a. Perform PCR using primers specific to the gene of interest and selectable marker to confirm the presence of the transgene.

Fungal Transformation Efficiency

1. **Efficiency Calculation:** Calculate transformation efficiency as the number of confirmed transformants per microgram of DNA used or per 10^6 spores.

Troubleshooting

1. **Low Transformation Efficiency:** Optimize spore concentration, induction conditions, and co-cultivation times.
2. **High Background:** Ensure proper antibiotic concentrations and use of cefotaxime to eliminate *Agrobacterium*.
3. **Poor Spore Germination:** Check spore viability and adjust incubation conditions.

This detailed protocol provides a comprehensive guide for performing Agrobacterium-mediated transformation in *Coleophoma empetri* and *Aspergillus nidulans*, facilitating the study of gene function and genetic manipulation in these fungi.

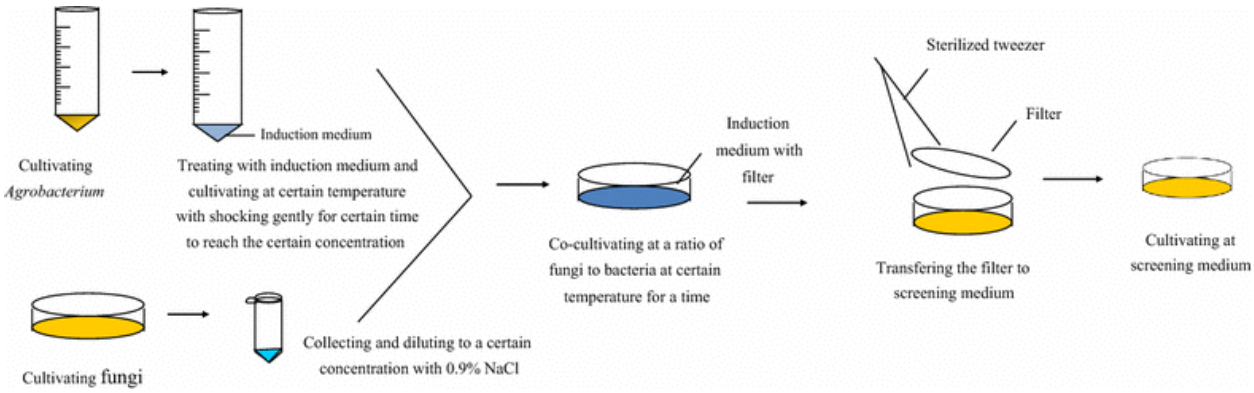

Figure 4: Agrobacterium-Mediated Transformation in Fungi[4]

5. Procedure

Amplification of Vector Base and 3' UTR Region

Vector Backbone Amplification: Amplification of the Vector Backbone, serving as the foundation for constructing the Gene X Deletion Cassette. This includes elements required for the insertion of the deletion cassette, such as promoters and selection markers. Amplification is accomplished through Polymerase Chain Reaction (PCR) using specific primers designed for the Vector Base.

[4] *Li, Dandan & Tang, Yu & Lin, Jun & Cai, Weiwen. (2017). Methods for genetic transformation of filamentous fungi. Microbial Cell Factories. 16. 168. 10.1186/s12934-017-0785-7.*

3' UTR Region Amplification: Simultaneously, the 3' Untranslated Region (3' UTR) of the gene of interest is amplified. This region follows the coding region of the gene and is vital for proper regulation. Amplification is achieved through PCR with primers tailored to the 3' UTR.

Fusion of Vector Backbone and 3' UTR Region

Infusion Cloning: The Infusion Cloning technique is employed to seamlessly fuse the amplified Vector Backbone and the 3' UTR Region. This fusion is achieved by meticulously designing primers for PCR amplification, ensuring overlapping sequences between the two fragments. (Figure 5). During fusion, these overlapping sequences facilitate the formation of a single, continuous DNA fragment comprising the Vector Base and the 3' UTR.

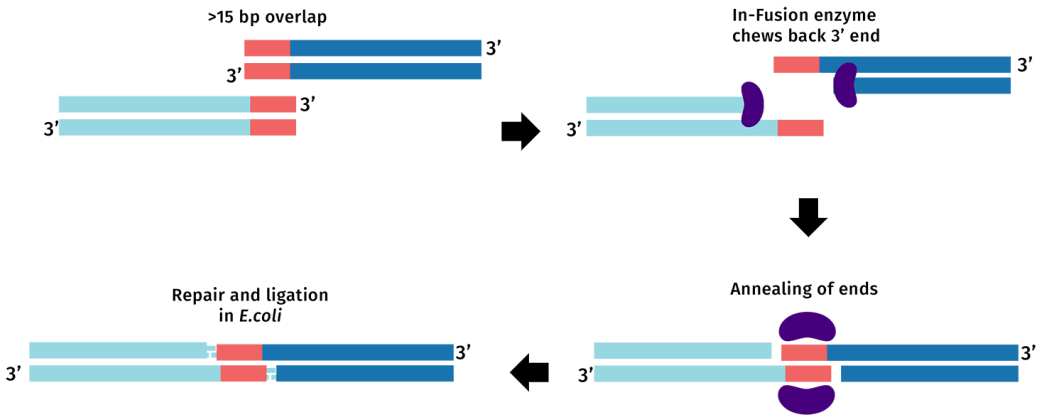

Figure 5: In-Fusion Cloning technique[5]

Selection and Transformation into E. coli via Agrobacterium-Mediated Transfer

1. To facilitate the selection of transformed constructs and their subsequent transformation, a pivotal aspect of the Gene X Deletion Cassette is the inclusion of a Kanamycin resistance gene within the Vector Backbone. The integration of the Gene X Deletion Cassette into E. coli is accomplished through a specialized method involving Agrobacterium-mediated transfer.
2. *Agrobacterium tumefaciens*, renowned for its natural ability to transfer genetic material, serves as a vector for transporting the plasmid containing the Gene X Deletion Cassette into the E. coli host. The transformed Agrobacterium, now containing the plasmid, is combined with E. coli cells. A co-cultivation process is initiated, allowing Agrobacterium to transfer the plasmid carrying the deletion cassette into the E. coli host. The plasmid integration is mediated by Agrobacterium's Ti plasmid, acting as a transfer agent. E. coli

[5] *In-Fusion Cloning - Snapgene. (n.d) " SnapGene, https://www.snapgene.com/guides/in-fusion-cloning. Accessed 15 January 2024*

cells are carefully incubated under conditions supporting plasmid expression and stable maintenance, enabling them to accept and host the Gene X Deletion Cassette. The selection plate consists of kanamycin, allowing only E. coli bacteria that have taken up the plasmid to grow.

Plasmid Isolation and Verification

Upon completion of the transformation, plasmid isolation becomes the subsequent crucial step. Plasmid isolation is imperative for further analyses. Following plasmid extraction, rigorous verification procedures are initiated. This involves Polymerase Chain Reaction (PCR) and confirmation of the Kanamycin resistance marker's presence within the plasmid. This dual verification ensures the successful integration of the plasmid into the host and the structural integrity of the plasmid itself.

Amplification of Fused Cassette

Post successful verification and isolation of the plasmid, the next stage of construction involves amplifying the isolated plasmid. Amplification yields a plasmid containing the fused 3' UTR and Vector Backbone.

Fusion of 5' UTR and 3' UTR Cassette

As part of the continued construction, the amplified plasmid, now encompassing the 3' UTR and Vector Backbone, is joined with the amplified 5' Untranslated Region (5' UTR). This fusion step is crucial as it completes the Gene X Deletion Cassette, incorporating all necessary regions for gene regulation and deletion.

Verification of Construct

In the verification stage, the fully assembled Gene X Deletion Cassette is transformed into E. coli bacteria using Agrobacterium transfer. Selection is achieved by cultivating the bacteria on a Kanamycin selection plate, allowing the identification of colonies that have successfully incorporated the plasmid carrying the deletion cassette. Following this selection, plasmid isolation is performed on bacterial colonies, and PCR is employed once again to confirm the presence and structural integrity of the deletion cassette, ensuring the correct construct has been obtained.

Transformation into Fungi for Gene Replacement

With the fully verified Gene X Deletion Cassette in hand, the final step involves introducing this cassette into fungi cells for precise gene replacement.

Fungi Transformation

The verified Gene X Deletion Cassette is transformed into fungi cells, with various fungi species employed based on specific research requirements.

Homologous Recombination for Gene Replacement

Inside the fungi cells, homologous recombination techniques are utilized, representing an advanced genetic process crucial for precise gene replacement. This process allows the removal of the target Gene X while integrating the Gene X Deletion Cassette in its place. The 5' UTR and 3' UTR regions, coupled with the regulatory Vector Backbone, collaborate to control gene expression, culminating in gene deletion and replacement.

Isolation of Fungus Colonies with Zeocin Selection

1. To selectively isolate fungi colonies that have undergone the desired gene replacement, Zeocin, an antibiotic selectable marker, is employed. Fungi cells that have integrated the Gene X Deletion Cassette and undergone successful gene replacement will exhibit resistance to Zeocin. These Zeocin-resistant colonies are isolated and serve as subjects for further research or applications.
2. The completion of these steps marks the culmination of the Gene X Deletion Cassette construction process and its successful integration into fungi for gene replacement, allowing for the precise manipulation of Gene X and the exploration of its regulatory role in a biological context.

6. Shake Flask Trials and HPLC Analysis

Concurrently with the construction of the Gene X Deletion Cassette, extensive Shake Flask (SF) trials were conducted to evaluate its impact on secondary metabolite production in various fungi strains.

1. **Experimental Setup:** Shake Flask trials involved cultivating fungi strains under controlled laboratory conditions in liquid media supplemented with nutrients conducive to secondary metabolite production. Each flask contained a specific fungal strain transformed with either the wild-type or the Gene X deletion cassette.

2. **Purpose of Shake Flask Trials:** The primary objective of these trials was to assess the influence of the Gene X Deletion Cassette on secondary metabolite biosynthesis. Shake Flask experiments provided a scalable and cost-effective means to simulate large-scale fermentation conditions, enabling the initial screening of fungal strains for enhanced secondary metabolite production.
3. **HPLC Analysis:** High-Performance Liquid Chromatography (HPLC) served as the primary analytical tool for quantitative analysis and profiling of the targeted secondary metabolites. Chromatographic data enabled the comparison between wild-type strains and those with the deleted Gene X, highlighting any significant changes in secondary metabolite production.

7. Results

The study successfully implemented the Gene X Deletion Cassette construction and evaluated its impact on secondary metabolite production:

1. ***Construction of Gene X Deletion Cassette:*** PCR and In-Fusion Cloning techniques effectively assembled the deletion cassette, validated through molecular verification methods.
2. ***Agrobacterium-Mediated Transformation:*** Co-cultivation with Agrobacterium facilitated the efficient transfer of the Gene X Deletion Cassette into E. coli, where its integration and stability were ensured under selective conditions.
3. ***Fungi Transformation and Gene Replacement:*** Optimized transformation protocols enabled the successful introduction of the deletion cassette into various fungi species, followed by homologous recombination for precise gene replacement.
4. ***Secondary Metabolite Production:*** Shake Flask trials demonstrated a significant increase in targeted secondary metabolite production in Gene X-deleted fungal strains compared to wild-type controls.
5. ***Quantitative Analysis:*** HPLC quantification revealed a production increase from 1.2 g/L in wild-type strains to 2.8 g/L in Gene X-deleted strains (Figure 6), highlighting the cassette's efficacy in enhancing secondary metabolite biosynthesis

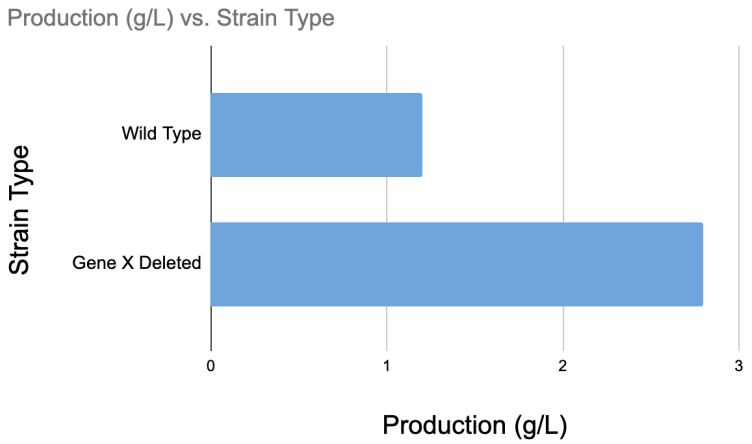

Figure 6: Comparative Increase in Secondary Metabolite Production: The wild-type strain exhibited a production level of 1.2 g/L, whereas the strain with the deleted Gene X achieved an impressive production of 2.8 g/L. This substantial improvement underscores the efficacy of the Gene X deletion cassette in positively influencing the overproduction of the targeted secondary metabolites.

8. Discussion

8.1 Mechanisms of Gene X-Mediated Repression

The mechanisms of Gene X-mediated repression involve the binding of Gene X to carbon catabolite repression elements (CCREs) in the promoters of target genes. This binding prevents the recruitment of the transcriptional machinery, thereby blocking gene expression. The deletion of gene X disrupts this repression, leading to the derepression of secondary metabolite biosynthetic genes and increased metabolite production.

8.2 Broader Implications of gene X Deletion in Fungal Biotechnology

The findings of this study have broader implications for fungal biotechnology. The deletion of gene X can be used as a strategy to enhance the production of valuable secondary metabolites in various fungal species. This approach can be applied to industrial-scale production processes, potentially increasing the yield and reducing the costs of producing antibiotics, mycotoxins, and other bioactive compounds.

8.3 Advantages and Challenges of In-Fusion Cloning

In-Fusion cloning offers several advantages, including high efficiency, the ability to clone large inserts, and the capacity to assemble multiple fragments in a single reaction. However, challenges remain, such as optimizing the conditions for each specific application and ensuring

the accuracy of the assembled constructs. Despite these challenges, In-Fusion cloning remains a powerful tool for constructing gene deletion cassettes and other genetic modifications in fungi.

9. Conclusion

The deletion of the gene X gene significantly enhances secondary metabolite production in fungi by relieving carbon catabolite repression. In-Fusion cloning was successfully employed to construct gene deletion cassettes, facilitating targeted genetic modifications in these fungi. The findings of this study have important implications for fungal biotechnology, offering new strategies for enhancing the production of valuable secondary metabolites.

10. Learning

The tenure of my internship at Biocon has been an intellectually enriching and professionally rewarding experience. The collective expertise of the seasoned professionals at Biocon has played a pivotal role in bridging the theoretical and practical aspects of my knowledge. This immersive experience has deepened my understanding of various molecular biology techniques employed in laboratory research.

Working collaboratively with a highly skilled team has been both insightful and enjoyable. I have had the privilege of acquiring a diverse range of indispensable laboratory skills. The challenges encountered during the internship have contributed significantly to the refinement of my self-confidence, providing exposure to a broad spectrum of techniques involved in strain improvement and screening. Additionally, this experience has sharpened my observational skills, cultivated an insatiably inquisitive mindset, and enhanced my communication and interpersonal skills.

While I have achieved considerable proficiency during this internship, the pursuit of a comprehensive understanding of the various facets of research remains ongoing. I am acutely aware of the necessity to further develop my public speaking abilities, a crucial aspect in the realm of scientific communication. The technical proficiency gained during this internship has significantly honed my analytical skills, laying a robust foundation for future endeavors in the field of research or within an industrial career.

In summary, my internship experience at Biocon has been exceptionally transformative, providing a rich tapestry of knowledge and skills that will undeniably prove invaluable in my future pursuits

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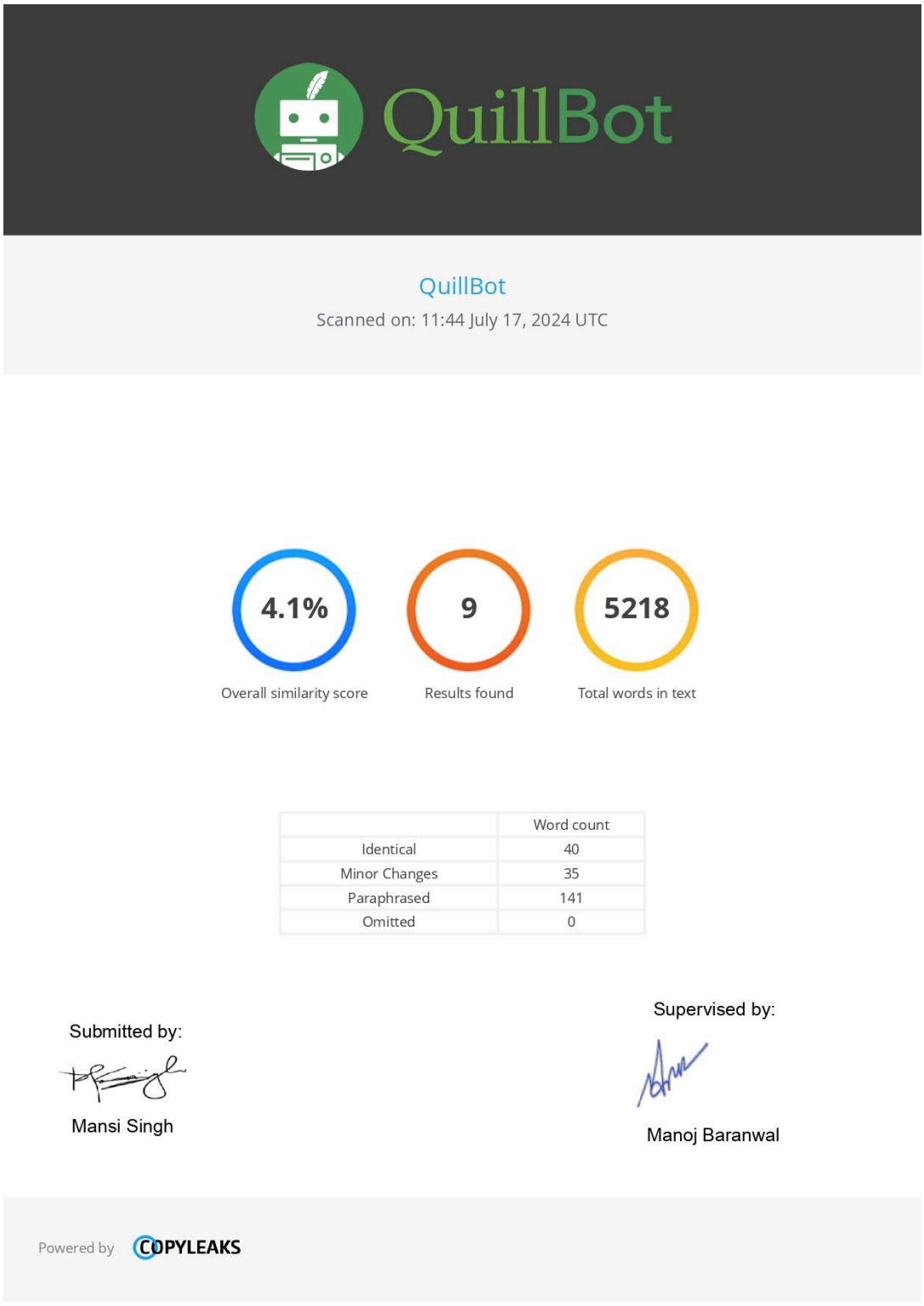

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