

Studies *in vivo* on the catabolism of gentisate and resveratrol in *Aspergillus niger*

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Abstract

Studies *in vivo* on the catabolism of gentisate and resveratrol in *Aspergillus niger*

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Aspergillus niger is a filamentous ascomycete fungus commonly found in soil and decomposing plant matter. It possesses numerous genes for primary and secondary metabolism and efficiently utilizes plant polysaccharides via a broad set of extracellular enzymes. Recently, sequenced fungal genomes, including *A. niger*, reveal gene models with amino acid sequences similar to bacterial compounds-degrading genes. Despite advances in genome sequencing, there remains a significant gap in the fundamental understanding of catabolic pathways for plant-derived aromatic compounds in fungi. Many of the genes encoding aromatic compound-degrading enzymes in fungi have not been identified, and in some cases, some of these pathway intermediates remain unknown in filamentous fungi. As part of an ongoing effort to understand the genes involved in the degradation of aromatics in *A. niger*. I have investigated *in vivo* the genes encoding enzymes in the pathway of two aromatic compounds, namely gentisate and resveratrol. In the first research chapter, I investigated the role of genes involved in the catabolism of gentisate in *A. niger* using multiple approaches of sequence homology to functionally characterize gentisate pathway genes in bacteria, as well as transcriptome analysis. This initially identified two gene clusters (*NRRL3_05234–NRRL3_05237* and *NRRL3_10244–NRRL3_10250*) encoding enzymes with potentially similar functions. Further investigation through gene knock-out studies, functional complementation test, and the detection of pathway intermediates using LC-MS identified genes in the first gene cluster (Δ *NRRL3_05234–NRRL3_05237*) as the genes that catalyze gentisate degradation in *A. niger*. Of note is the identification of a gene (*miaA*) with no homology to known bacteria gentisate pathway gene but which was found to be indispensable in the catabolism of gentisate through the conversion of maleylpyruvate to fumarylpyruvate. With these approaches, I was able to confirm and refine the assignment of these genes and their roles in the catabolism of gentisate in *A. niger*. The second research chapter is the result of the attempt to assign roles to the second gene cluster

(*NRRL3_10244- 10250*). Using similar approaches as stated above, excluding the functional complementation tests and transcriptome analysis, I identified the genes and their role in the resveratrol catabolic pathway in *A. niger*.

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Dedication

This thesis is lovingly dedicated to the memory of my beloved father, Mr. Omoruyi Oviasuyi, whose unwavering belief in the power of education, especially for the girl child, continues to echo through every step of my journey.

Every page of this book captures your voice, values, and dreams, even when you are no longer physically here. Your unyielding support, relentless sacrifices, and quiet but powerful determination that I could accomplish anything if I pursued it with diligence and passion set the groundwork for what I have become.

Apart from being a father, you were my strongest supporter, educator, and protector. Sometimes, I doubted myself, and you believed in me. You never wavered in your belief that I belonged in each classroom, lab, or academic hall, regardless of their distance from home

Your vision, your love, and your refusal to let me dream small carried me here.

Every achievement of this Ph D, every long night, every discovery, every challenge overcome, is as much yours as it is mine. I carry your legacy with pride and gratitude, and I hope this work stands as a small reflection of the greatness you saw in me.

Thank you, Daddy, for everything. This is for you. Continue to rest on Papa.

Contribution of Authors

Chapter 1: Introduction

This chapter provides a foundational overview that informs the discussions and analyses presented in the subsequent chapters. Idowu Bello-Osagie compiled the writing, and Dr. Adrian Tsang reviewed and edited it. All Figures were created by Idowu Bello-Osagie, except for Figure 1.1, which was modified and taken from Phale *et al.* (2020) and Figure 1.7, from Ouedraogo *et al.* 2020. (Copyright permission was not required).

Chapter 2: Functional characterization of gentisate catabolism in *Aspergillus niger* reveals a gene encoding a CENP-V/GFA domain-containing protein is responsible for the conversion of maleylpyruvate to fumarylpyruvate

In this chapter, we employed multiple approaches to identify genes involved in the catabolism of gentisate in *A. niger*. Dr. Justin Powlowski first predicted the pathway genes and gifted us with the purified gentisate 1,2-dioxygenase and the plasmid-cloned maleylpyruvate isomerase gene from *Ralstonia* species used in this study.

Bioreactor cultivations with *Aspergillus niger* on gentisate and fructose as a reference compound were prepared by Mark Arentshorst. The transcriptome data from bioreactor cultivations of *A. niger* were analyzed by Dr. Ian Reid. Analysis of accumulated metabolites in pathway mutants was performed by Dr. Marcos DiFalco using mass spectrometry. Michael Sgro performed protein modelling. Gene deletion, PCR validation, preparation of growth media for phenotypic study and manuscript preparation were done by Idowu Bello-Osagie with contributions from Michael Sgro. Dr. Adrian Tsang and Dr. Arthur F. J. Ram reviewed and edited the manuscript. (Manuscript in preparation)

Chapter 3: Functional characterization of resveratrol catabolic pathway in *Aspergillus niger*

The project began with the objective of investigating two gene clusters; both were initially predicted to be involved in gentisate degradation. Through a combination of comparative genomics

and targeted gene deletions, we were able to functionally characterize that one of the clusters encodes the enzymes required for gentisate catabolism in *Aspergillus niger* (Chapter 2), and the second gene cluster is involved in resveratrol catabolism. Experiments, including gene knock-out, PCR validation and phenotypic studies, were performed by Idowu Bello-Osagie. Mass spectrometry analysis of accumulated metabolites in pathway mutants was performed by Dr. Marcos DiFalco. The manuscript was written by Idowu Bello-Osagie, and Dr. Adrian Tsang reviewed and edited the manuscript. (Manuscript in preparation)

Chapter 4: Conclusions and future work

Chapter four summarizes the main findings of the thesis and discusses the implications for the work and future directions.

Table of Contents

List of Figures.....	xiii
List of Supplementary Figures	xvi
List of Abbreviations.....	xviii
Chapter 1: Introduction.....	1
1.1 Plant-derived secondary metabolites	1
1.2 Classification of plant-derived secondary metabolites.....	1
1.2.1 Terpenes	1
1.2.2 Phenolics	3
1.2.3 Tannins.....	5
1.2.4 Alkaloids	7
1.3 Lignin.....	9
1.4 Catabolism of aromatic compounds in plants	11
1.4.1 The seven central aromatic intermediates.....	14
1.4.2 Convergence of the catechol, protocatechuate, and hydroxyquinol branches into the β -Ketoadipate pathway	15
1.4.3 Hydroquinone degradation pathway in bacteria and fungi	18
1.4.4 Catabolism of gallate in bacteria and fungi.....	20
1.4.5 Catabolism of pyrogallol in bacteria and fungi	21
1.5 Gentisate and resveratrol.....	22
1.5.1 Gentisic acid (2,5-dihydroxybenzoic acid (2,5-DHB)).....	23
1.5.2 Resveratrol	25
1.6 <i>Aspergillus niger</i> as a model organism	27
1.7 Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas).....	28
1.8 Rationale for the study	30
1.9 Specific goal of the study	32
References.....	33
Chapter 2: Functional characterization of gentisate catabolism in <i>Aspergillus niger</i> reveals a gene encoding a CENP-V/GFA domain-containing protein is responsible for the conversion of maleylpyruvate to fumarylpyruvate.....	53
2.1 Abstract.....	53
2.2 Introduction	54
2.3 Results.....	58

2.3.1 Candidate genes of the gentisate pathway based on orthology and transcriptome analysis	58
2.3.2 The orthologues of the bacterial maleylpyruvate isomerase are not essential for the catabolism of gentisate	63
2.3.3 Cluster of genes involved in gentisate catabolism.....	64
2.3.4 Loss-of-function of <i>NRRL3_05235</i> can be functionally complemented by maleylpyruvate isomerase genes.....	71
2.3.5 <i>NRRL3_05237</i> is required for the catabolism of gentisate	72
2.4 Discussion.....	79
2.5 Materials and Methods.....	85
2.5.1 Chemicals	85
2.5.2 Strains, media and conditions of growth.....	85
2.5.3 Bioreactor cultivation	86
2.5.4 RNA sequencing and transcriptome analysis.....	87
2.5.5 Construction of <i>A. niger</i> mutant strains using CRISPR/Cas9.....	87
2.5.6 Transformation of <i>A. niger</i> and verification of mutant strains.....	87
2.5.7 Construction of gene replacement cassettes for Δ <i>NRRL3_05235</i> in <i>A. niger</i>	88
2.5.8 Metabolite analysis from mutant strains using liquid chromatography-mass spectrometry (LC-MS)	88
2.5.9 Generation of maleylpyruvate.....	89
2.5.10 Protein modelling.....	89
2.6 Data availability.....	90
2.7 Funding and additional information.....	90
2.8 Conflict of interest	90
2.9 Supplementary Figures	90
Chapter 3: Functional characterization of resveratrol catabolic pathway in <i>Aspergillus niger</i>	113
3.1 Abstract.....	113
3.2 Introduction	114
3.3 Results and Discussion	118
3.3.1 Predicted function of the <i>NRRL3_10244–NRRL3_10250</i> gene cluster of <i>A. niger</i> ...	118
3.3.2 <i>NRRL3_10247</i> encodes resveratrol cleavage dioxygenase <i>RcoA</i>	121
3.3.3 <i>NRRL3_03887</i> (<i>vdhA</i>) encodes a versatile benzaldehyde dehydrogenase.....	122
3.3.4 Δ <i>NRRL3_10245</i> mutant accumulates 3,5-dihydroxybenzoate.....	131
3.3.5 Δ <i>NRRL3_10244</i> accumulates 2,3,5-trihydroxybenzoic acid	134
3.3.6 Δ <i>NRRL3_10248</i> results in severe growth impairment.....	138
3.3.7 Δ <i>NRRL3_10246</i> encodes the regulator of the resveratrol gene cluster	141
3.3.8 Conversion of 4-hydroxybenzoate to protocatechuate	141
3.3.9 Phenotypes of 3,4-DHB mutants on 4-hydroxybenzaldehyde and 4-hydroxybenzoate	142
3.4 Conclusion	144

3.5 Materials and Methods.....	145
3.5.1 Chemicals.....	145
3.5.2 Strains, media and conditions of growth.....	145
3.5.3 Guide sequence selection and validation	147
3.5.4 Construction of gene deletion cassettes using CRISPR/Cas9.....	147
3.5.5 Transformation, mutant purification, and screening protocol.....	147
3.5.6 Identification of an additional homologue from related fungal species	148
3.6 Metabolite profiling by liquid chromatography-mass spectrometry.....	148
3.6.1 Sample preparation	148
3.6.2 Liquid chromatography-mass spectrometry (LC-MS) analysis	148
3.7 Data analysis.....	149
3.7.1 Quantification methodology.....	149
3.8 Data availability.....	149
3.9 Funding and additional information.....	149
3.10 Conflict of interest	149
3.11 Supplementary Figures	150
References.....	158
Chapter 4: Comprehensive insights into the gentisate (2,5-DHB) and resveratrol catabolic pathways in <i>Aspergillus niger</i>: Conclusions and future direction	164
4.1 Gentisate catabolic pathway	164
4.2 Resveratrol catabolic pathway.....	164
4.3 Implications and future directions.....	166
4.4 Conclusion	168

List of Figures

Figure 1.1. Plant aromatic compounds funnelled into seven central metabolic pathways.....	15
Figure 1.2. The bacterial and fungal metabolic pathways for protocatechuate, catechol, and hydroxyquinol converge into the β -ketoadipate Pathway.....	17
Figure 1.3. Pathways for the biodegradation of hydroquinone under aerobic conditions.....	19
Figure 1.4. The proposed pathway for the degradation of gallotannins by <i>Citrobacter freundii</i>	22
Figure 1.5. Catabolic pathway for gentisate conversion to pyruvate, maleate, and fumarate in bacteria.....	24
Figure 1.6. Proposed pathway for resveratrol degradation in fungi and bacteria.....	27
Figure 1.7. Genome editing by CRISPR-Cas systems.....	30
Figure 2.1. Catabolic pathway for the gentisate conversion to pyruvate, maleate, and fumarate in bacteria.....	56
Figure 2.2. Organization of genes encoding predicted gentisate catabolic enzymes and co-localized, upregulated genes.....	62
Figure 2.3. Growth phenotype of <i>A. niger</i> parent strain (CBS 138852) and predicted maleylpyruvate isomerase deletion mutants.....	64
Figure 2.4. Growth phenotypes of <i>A. niger</i> parent strain (CBS 138852) and predicted gentisate cluster deletion mutants.....	65
Figure 2.5 Growth phenotypes of <i>A. niger</i> parent strain (CBS 138852), with co-localized pathway genes, Δ <i>gtaR</i> (Δ NRRL3_05233) and Δ NRRL3_05234.....	66
Figure 2.6 Growth phenotype and gentisate utilization of <i>Aspergillus niger</i> CBS 138852 and the gentisate 1,2-dioxygenase mutant Δ <i>gdoA</i> (Δ NRRL3_05236).....	67
Figure 2.7. Growth phenotype and metabolic profiling of <i>Aspergillus niger</i> CBS 138852 and the deletion mutant Δ <i>miaA</i> (Δ NRRL3_05235)	69
Figure 2.8. LC-MS analysis of culture supernatants from Δ <i>miaA</i> (Δ NRRL3_05235) and <i>A. niger</i> parent strain (CBS 138852) grown on gentisate	70
Figure 2.9. LC-MS/MS fragmentation of the pathway intermediate, compared to the reference compound.....	71

Figure 2.10. Growth phenotype of functionally complemented $\Delta miaA$ (NRRL3_05235) strains in <i>A.niger</i>	72
Figure 2.11. Growth phenotype and gentisate catabolism in <i>Aspergillus niger</i> parent strain (CBS 138852) and the predicted fumarylpyruvate hydrolase deletion mutant $\Delta fphA$ (Δ NRRL3_05237).....	74
Figure 2.12. LC-MS Analysis of culture supernatants from $\Delta fphA$ (Δ NRRL3_05237) and <i>A. niger</i> parent strain (CBS 138852) grown on gentisate.....	75
Figure 2.13. Growth phenotype of disrupted $\Delta miaA$ replaced with all MPIs in $\Delta fphA$ (Δ NRRL3_05237) strains.....	76
Figure 2.14. LC-MS analysis of gentisate utilization in $\Delta fphA$ (Δ NRRL3_05237) strains with $\Delta miaA$ replaced with MPIs.....	78
Figure 2.15. Molecular structure correlator identification of fumarylpyruvate as the best match to the MS/MS fragmentation profile.....	79
Figure 2.16. Comparative organization of gentisate catabolic pathway genes in <i>Aspergillus niger</i> and <i>Candida parapsilosis</i>	80
Figure 2.17. Pathway for the catabolism of gentisate in <i>A.niger</i>	84
Figure 3.1. Proposed pathway for resveratrol degradation in fungi.....	117
Figure 3.2. Gene cluster organization for predicted resveratrol catabolism in <i>Aspergillus species</i>	121
Figure 3.3. Growth phenotype of <i>A. niger</i> parent strain (CBS 138852) and resveratrol pathway mutants	122
Figure 3.4. LC-MS detection of the accumulation of metabolites in $\Delta rcoA$ (Δ NRRL3_10247)	123
Figure 3.5. Growth phenotype of <i>A. niger</i> mutants on hydroxybenzene compounds....	125
Figure 3.6. LC–MS profiles of $\Delta vdhA$ (Δ NRRL3_03887) and parent strain grown on resveratrol.....	127
Figure 3.7. Accumulation of 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde by $\Delta vdhA$ (Δ NRRL3_03887).....	129
Figure 3.8. Comparison of MS/MS fragmentation spectra of reference hydroxybenzaldehydes and compounds accumulated in $\Delta vdhA$	131

Figure 3.9. LC–MS profiles of $\Delta dbaA$ ($\Delta NRRL3_10245$) and parent strain grown on resveratrol	133
Figure 3.10 Accumulation of 3,5-DHB by $\Delta dbaA$ ($\Delta NRRL3_10245$).....	134
Figure 3.11. MS/MS fragmentation pattern of 3,5-DHB compared to the accumulated metabolite in the $\Delta dbaA$ ($\Delta NRRL3_10245$) strain	135
Figure 3.12. LC-MS analysis of pathway intermediate detected in $\Delta tdoA$ ($\Delta NRRL3_10244$) grown on resveratrol.....	137
Figure 3.13 Analysis of resveratrol consumption and pathway intermediate by LC-MS:.....	138
Figure 3.14. Accumulation of a low-intensity intermediate in the $\Delta ophA$ ($\Delta NRRL3_10248$) strain.....	140
Figure 3.15. LC-MS analysis of resveratrol utilization in $\Delta ophA$ ($\Delta NRRL3_10248$) strain.....	141
Figure 3.16. Pathway for the degradation of Resveratrol.....	144

List of Supplementary Figures

Figure S2.1. PCR verification of $\Delta NRRL3_04710$ ($\Delta mpiA$).....	91
Figure S2.2. PCR verification of $\Delta NRRL3_09971$ ($\Delta mpiB$).....	92
Figure S2.3. PCR verification of $\Delta NRRL3_04710$, $\Delta NRRL3_09971$ and $\Delta NRRL3_04710/\Delta NRRL3_09971$ in $\Delta NRRL3_09971$ strain.....	93
Figure S2.4. PCR verification of $\Delta NRRL3_05234-05237$	94
Figure S2.5. PCR verification of $\Delta NRRL3_10244-NRRL3_10250$	95
Figure S2.6. PCR verification of $\Delta NRRL3_05233$ ($\Delta gtaR$).....	96
Figure S2.7. PCR verification of $\Delta NRRL3_05234$	97
Figure S2.8. PCR PCR verification of $\Delta NRRL3_05236$ ($\Delta gdoA$).....	98
Figure S2.9. PCR verification of $\Delta NRRL3_05235$ ($\Delta miaA$).....	99
Figure S2.10. PCR verification of $\Delta NRRL3_05237$ ($\Delta fphA$).....	100
Figure S2.11 Structural alignment of AlphaFold-predicted maleylpyruvate isomerase proteins from <i>A. niger</i> with bacterial homolog.....	101
Figure S2.12 Structural alignment of bacterial MPIs.....	102
Figure S2.13 Structural alignment of AlphaFold-predicted maleylpyruvate isomerase protein from <i>A. niger</i> with bacterial homolog.....	103
Figure S2.14 Growth phenotype of predicted pathway mutants on various aromatic compounds.....	104
Figure S3.1. Diagram of mutant generation and verification of $\Delta NRRL3_10244$	151
Figure S3.2. Diagram of mutant generation and verification of $\Delta NRRL3_10245$	152
Figure S3.3. Diagram of mutant generation and verification of $\Delta NRRL3_10246$	153
Figure S3.4. Diagram of mutant generation and verification of $\Delta NRRL3_10247$	154
Figure S3.5. Diagram of mutant generation and verification of $\Delta NRRL3_10248$	155
Figure S3.6. Diagram of mutant generation and verification of $\Delta NRRL3_03887$	156
Figure S3.7. Diagram of mutant generation and verification of $\Delta NRRL3_10244$ - $NRRL3_10250$	157
Figure S3.8. Diagram of mutant generation and verification of $\Delta NRRL3_05234$ - $NRRL3_05237$	158

List of Tables

Table 2.1. Sequence identity of <i>A. niger</i> proteins to orthologues of bacterial gentisate catabolic enzymes.....	59
Table 2.2. Pairwise sequence identity (%) between maleylpyruvate isomerases and their orthologues in <i>A. niger</i>	60
Table 2.3. RNA-sequencing data for predicted gentisate degradation pathway genes in <i>A. niger</i>	61
Table 2.4. <i>Aspergillus niger</i> strains used in this study.....	86
Table 3.1. <i>A. niger</i> strains used in this study.....	146

List of Abbreviations

ADO: aromatic-ring cleavage dioxygenase
ARH: aromatic-ring hydrolase
CCO: carotene-cleaving oxygenase
FAA: fumarylacetoacetase family protein
FTF: fungal-specific transcription factor
GFA: glutathione-dependent formaldehyde-activating family protein
GDO: gentisate 1,2-dioxygenase
MPI: maleylpyruvate isomerase
ZAD: zinc-type alcohol dehydrogenase
2,5-DHB: 2,5-dihydroxybenzoic acid
2-HB: 2-hydroxybenzoic acid, salicylate
2,3-DHB: 2,3-dihydroxybenzoic acid
3-HB: 3-hydroxybenzoic acid
3,5-DHBZA: 3,5-dihydroxybenzaldehyde
4-HBZA: 4-hydroxybenzaldehyde
3,5-DHB: 3,5-dihydroxybenzoic acid
2,3,5: THB (2,3,5-trihydroxybenzoic acid
ALD: aldehyde dehydrogenase
BLASTP: protein basic local alignment search tool
UV-Vis: Ultraviolet-Visible Spectroscopy
EXP: inferred from experiment
IEP: inferred from expression pattern
IMP: inferred from mutant phenotype
ISO: inferred from sequence orthology
LC: liquid chromatography
MS: mass spectrometry
TCA: Tricarboxylic acid
TPM: transcripts per million
HPLC: High-performance liquid chromatography

Chapter 1: Introduction

1.1 Plant-derived secondary metabolites

Plant-derived secondary metabolites form a diverse range of organic compounds. These metabolites are produced in response to abiotic and biotic stimuli, have immense chemical and biological diversity, and are typically localized in specific tissues or specialized organs (Sharma & Sharma, 2022). While they do not directly participate in essential plant life processes like photosynthesis or nutrient assimilation (González-Mera *et al.*, 2019), they contribute to plant growth, environmental adaptation, defense response, and protection from environmental stress (Şeker & Erdoğan, 2023). Furthermore, these metabolites help repel pests and pathogens and support the mutualistic association between microbial communities and plants (Guerrieri *et al.*, 2019). Additionally, they act as allelopathic agents, impacting the growth, survival, and reproduction of neighbouring plants, attracting seed pollinators, and aiding plants in adapting to abrupt changes in environmental conditions (Ramakrishna & Ravishankar, 2011). Many secondary metabolites of plants also exhibit beneficial effects on human health (Ullrich *et al.*, 2019).

1.2 Classification of plant-derived secondary metabolites

Plant-derived secondary metabolites are commonly classified according to their biosynthetic pathways into terpenes, phenolics and alkaloids.

1.2.1 Terpenes

In plants, terpenes represent the largest and most diverse class of secondary metabolites (Roba, 2020), and they serve as the building blocks for countless essential plant molecules. They are constructed from five-carbon isoprene units (C_5H_8), which combine in various configurations to create molecules of different sizes and complexities (Harmange-Magnani *et al.*, 2020). Their structural diversity is remarkable, with over 50,000 different compounds identified to date, making them the largest class of plant secondary metabolites (Xavier *et al.*, 2023).

Terpenes serve numerous vital functions as they act as defensive compounds against herbivores and pathogens, with some having potent antimicrobial properties (Tiku, 2018). Terpenes, particularly low-molecular-weight monoterpenes and some sesquiterpenes, are often volatile organic compounds that contribute to plant aromas and play roles in attracting pollinators and beneficial insects while deterring harmful ones (Boncan *et al.*, 2020). Some also function as allelopathic agents, inhibiting the growth of competing plants in their vicinity (Verdeguer *et al.*, 2020).

The industrial and pharmaceutical significance of terpenes cannot be overstated, as many terpene-derived compounds have medicinal properties. For example, taxol, a diterpene from yew trees, is a potent anticancer drug, while artemisinin, a sesquiterpene from sweet wormwood, is used in malaria treatment (Alamgir & Alamgir, 2018). In some industries, terpenes are essential components of essential oils and perfumes (Sharmeen *et al.*, 2021). The growing biofuel industry is also exploring terpenes as potential renewable energy sources, as some of these compounds have properties similar to conventional petroleum-based fuels (Beller *et al.*, 2015).

Recent advances in biotechnology have enabled the engineering of microorganisms to produce valuable terpenes, offering more sustainable production methods compared to traditional plant extraction (Marienhagen & Bott, 2013). This has opened new possibilities for the large-scale production of terpene-based compounds with applications ranging from pharmaceuticals to industry. The environmental factors affecting terpene production in plants are complex and include light intensity, temperature, water availability, and stress factors (Kopaczyk *et al.*, 2020). Understanding these factors is necessary for optimizing the production of desired terpene compounds in natural plant systems or biotechnological applications.

Terpenes are systematically classified based on the number of isoprene units in their molecular structure, creating a diverse spectrum of compounds with distinct properties and biological functions. Following the isoprene rule, the classification is based on the number of isoprene (C_5) units in the molecule. Hemiterpenes (C_5), the simplest form containing a single isoprene unit, exemplified by isoprene (Wong, 2017). Monoterpenes, containing two isoprene units (C_{10}), include familiar compounds like limonene in citrus fruits, menthol in peppermint, and

pinene in pine resin. They serve ecological roles in pollinator attraction and herbivore deterrence (Harrewijn *et al.*, 2000).

Sesquiterpenes (C_{15}), formed from three isoprene units, encompass medicinally significant compounds, including artemisinin, a potent antimalarial drug derived from *Artemisia annua*, caryophyllene, found in black pepper, with anti-inflammatory properties and compounds like farnesol (Maurya *et al.*, 2021). The classification extends to diterpenes (C_{20}), which contain four isoprene units and include the anticancer drug taxol from the Pacific yew tree, as well as gibberellins that regulate plant growth and development, and phytol, essential for chlorophyll biosynthesis (Brahmkshatriya & Brahmkshatriya, 2013; Talapatra & Talapatra, 2015). Triterpenes (C_{30}) are composed of six isoprene units, and they play vital roles in cellular membrane structure and stability and include compounds like squalene, a precursor to sterols and hormones, and ursolic acid, found in apples and rosemary, with notable antioxidant properties (Lukić *et al.*, 2021).

Tetraterpenes (C_{40}), built from eight isoprene units, comprise pigment molecules. These include the carotenoids beta-carotene, a vitamin A precursor; lycopene, a non-provitamin A carotenoid; and the xanthophylls lutein and zeaxanthin. These compounds are involved in photosynthesis and photoprotection, contributing to the characteristic colours of many fruits and vegetables (Mosquera *et al.*, 2021). The classification culminates with polyterpenes, which consist of long chains of isoprene units and are exemplified by natural rubber ($(C_5H_8)_n$) derived from latex, providing structural and mechanical support to plants (Rea & Patel, 2017). This hierarchical classification system not only reflects the structural complexity of terpenes but also demonstrates their diverse biological functions, ranging from primary metabolism and cellular structure to ecological interactions and defense mechanisms, making them indispensable components of plant biology and valuable resources for human applications in medicine, industry, and agriculture.

1.2.2 Phenolics

Phenolics are a large and diverse class of secondary metabolites characterized by one or more hydroxyl groups attached to an aromatic ring (Wang *et al.*, 2020). They exhibit diverse biological activities, including antioxidant, anti-inflammatory, and disease-preventive effects and their health benefits are influenced by bioavailability, metabolism, and other physiological factors

(Ávila-Román *et al.*, 2021). Phenols are primarily synthesized through the shikimate pathway and phenylpropanoid metabolism, and they are ubiquitous in plants, contributing to growth, development, reproduction, and defense (Kumar *et al.*, 2023). Their structural diversity, from simple phenols to complex polymeric compounds, underlies their vast biological functions and applications in different industries (Zagoskina *et al.*, 2023). Their diverse structural properties and biological activities make them invaluable resources for commercial and therapeutic applications. Phenolic compounds play roles in drug development and therapeutic treatments, with resveratrol supporting cardiovascular health and curcumin exhibiting anti-inflammatory properties.

The agricultural sector has recognized the potential of phenolic compounds in crop protection and management; for example, tannins and flavonoids can be used as natural pesticides and disease-resistance enhancers. New applications for phenolic compounds, particularly in emerging fields like nanotechnology and sustainable materials, are being explored (Rosales & Fabi, 2023). The ability of phenolic compounds to act as reducing agents in the synthesis of nanoparticles and their potential as natural alternatives to synthetic polymers are areas of active investigation (Mondejar-Lopez *et al.*, 2024). As consumers' demand for natural products increases and environmental concerns grow, the role of phenolic compounds in industrial applications is likely to expand further.

The classification of phenolic compounds begins with simple phenols, which contain a single aromatic ring with hydroxyl groups. Hydroquinone and catechol, examples of phenolic compounds, demonstrate significant antioxidant and antimicrobial properties (Vuolo *et al.*, 2019). The "phenol" basic structure is a building block for more complex phenolic compounds. Phenolic compounds are broadly classified into simple and polyphenolic compounds (Al Mamari, 2021). Phenolic compounds with a singular phenol unit or its derivative, characterized by a C₆ skeletal structure, are termed simple phenolics. These compounds include hydroxyphenols, dihydroxybenzenes, trihydroxybenzenes, and pyrogallol (Al Mamari, 2021). Phenols with carboxylic functional (-COOH) groups are referred to as phenolic acids, which could either be hydroxybenzoic acid (when the -COOH group is directly bonded to the phenol ring) or hydroxycinnamic acids (when a three-carbon side chain containing a double bond separates the -COOH group from the phenol ring). Among hydroxybenzoic acids, gallic acid, abundant in grapes

and tea, demonstrates potent antioxidant activity, contributing to cellular protection and longevity. Hydroxycinnamic acids, such as caffeic and ferulic acids, enhance plant resilience by mitigating oxidative stress and acting as natural UV protectants, thereby protecting plant tissues from environmental damage, as demonstrated in several studies (Al Mamari, 2021; Ávila-Román *et al.*, 2021; Albuquerque *et al.*, 2021).

Aside from the earlier described classification scheme, phenolic compounds have also been classified according to de la Rosa *et al.* (2019) as flavonoids and non-flavonoids or monomeric or polymeric compounds, depending on their complexities. Flavonoids constitute one of the largest and most significant groups of phenolic compounds, characterized by their flavan nucleus structure (Heim *et al.*, 2002). They include compounds like anthocyanins (responsible for the vibrant colours in flowers and fruits), cyanidin, and delphinidin, which provide red, blue, and purple pigmentation (Brouillard & Dangles, 2017).

1.2.3 Tannins

Tannins are phenolic compounds with molecular masses typically ranging from 0.5 – 30 kilodaltons (Serrano *et al.*, 2009). The structural complexity of tannins directly influences their functionality, predominantly in their ability to form hydrogen bonds and engage in chemical interactions with proteins, carbohydrates, and metal ions (Engstrom *et al.*, 2022).

Hydrolyzable tannins are characterized by a central polyol core (typically glucose) esterified with gallic acid or its derivatives, and the ester bonds in these molecules can be cleaved through enzymatic activity, acid hydrolysis, or alkaline conditions, yielding smaller phenolic compounds (Koleckar *et al.*, 2008). Gallotannins, exemplified by tannic acid found in oak galls, represent one of the simplest forms of hydrolyzable tannins (Nollet & Toldra, 2012). Its molecular structure comprises multiple gallic acid units attached to a glucose core through ester linkages. More complex variants of hydrolyzable tannins include ellagitannins (where additional oxidative coupling between gallic acid units creates more sophisticated molecular architectures), as seen in the compound, punicalagin, from pomegranate (Mates *et al.*, 2024).

Aside from hydrolyzable tannins, a second category known as condensed tannins or proanthocyanidins exhibits fundamentally different chemical characteristics (Ávila-Román *et al.*, 2021). These compounds are formed through the condensation of flavan-3-ol units, primarily epicatechin and catechin, resulting in complex polymeric structures that resist hydrolysis. The polymerization process involves the formation of carbon-carbon bonds between individual flavanol units, resulting in chains of varying lengths and branching patterns (Xie & Dixon, 2005). This structural characteristic contributes to their stability and their distinct biological activities. Common examples include the procyanidins found in grape seeds and cocoa, which contribute to the astringent sensation experienced when consuming these products (Xie & Dixon, 2005).

Phlorotannins, primarily found in marine brown algae, represent a unique subset of tannins with distinct structural features and are polymers of phloroglucinol units connected through different types of chemical bonds, creating diverse architectural arrangements (Catarino *et al.*, 2022). Their presence in marine organisms highlights the evolutionary adaptation of tannin-like compounds across different ecological niches (Mates *et al.*, 2024).

In plants, tannins function as an advanced defense mechanism against herbivores and pathogens (Serrano *et al.*, 2009). The protein-binding capability of tannins can reduce the digestibility of plant tissues, making them less attractive to potential predators. Additionally, tannins exhibit antimicrobial properties, helping plants resist pathogenic organisms. This defensive role is particularly evident in young leaves and developing fruits, which often exhibit the highest tannin concentrations (Das *et al.*, 2020).

Tannins have found industrial applications across multiple sectors. Their ability to form stable crosslinks with collagen proteins in leather tanning transforms animal hides into durable leather (Ramshaw & Madhan, 2024). Tannins in the food industry are utilized as natural preservatives and flavor components, especially in beverages like tea and wine (Molino *et al.*, 2020). Their astringent properties contribute to the “characteristic mouthfeel” of these products while also serving as practical preservatives (Soares *et al.*, 2020). Their ability to inhibit microbial growth helps extend the shelf life of food products. In beverages, particularly wines, teas, and coffees, tannins contribute significantly to the sensory experience. They create the characteristic

astrigent sensation and complex flavour profiles that consumers associate with these beverages (Gonzalez-Munoz *et al.*, 2022). The interaction between tannins and proteins in saliva produces the dry, puckering sensation that is particularly noticeable in red wines (Kuhlman *et al.*, 2024). The environmental applications of tannins have gained attention in recent years, particularly in water treatment. Their ability to chelate heavy metals and bind to pollutants makes them effective natural agents for water purification, as demonstrated in original studies (Anjos *et al.*, 2024). This application offers an environmentally friendly alternative to conventional chemical treatment methods.

The health implications of tannins have garnered considerable scientific interest as research has revealed their potential as anti-inflammatory agents and antioxidants, and as modulators of biological processes (Bouarab Chibane *et al.*, 2019; Molnar *et al.*, 2024). Their protein-binding properties may influence physiological processes, while their ability to scavenge free radicals and chelate metal ions contributes to their antioxidant activity (Cano *et al.*, 2020). Studies have shown their potential benefits in areas ranging from cardiovascular health to cancer prevention, though many of these effects are still under investigation (Kleszcz *et al.*, 2023; Marrone *et al.*, 2023).

Recent technological advances have expanded the potential applications of tannin. Their use in sustainable materials, including biodegradable plastics and environmentally friendly adhesives, represents an emerging area of research (Ferri *et al.*, 2023). The development of new extraction and processing methods has also improved the efficiency of tannin utilization in traditional applications, while opening possibilities for novel uses (Cano *et al.*, 2020).

1.2.4 Alkaloids

Alkaloids are organic molecules containing nitrogen, with at least one nitrogen atom in a heterocyclic ring. They can be grouped based on their fundamental chemical structures as aromatics, acridones, carbolines, ergots, imidazoles, ephedras, manzamines, indoles, bisindoles, indolizidines, oxindoles, quinolines, phenyl isoquinolines, quinazolines, phenylethylamines, purines, piperidines, pyrrolidones, pyrrolidines, simple tetrahydroisoquinolines, pyrrolizidines, and pyridines (Tadeusz, 2015).

Alkaloids are classified based on a multiplicity of approaches, including their chemical structure, biosynthetic origin, or biological function. Based on biosynthesis, true alkaloids like quinine, caffeine, and morphine, which comprise nitrogen atoms within a heterocyclic ring, are obtained from amino acids. The protoalkaloids retain nitrogen but lack incorporation into a heterocyclic ring, e.g. ephedrine and colchicine, which are useful therapeutics. On the other hand, the pseudoalkaloids looked like true alkaloids in structure and function, but they were not derived from amino acids. Notable examples are solanine and cucurbitacins, which exhibit toxicity toward herbivores (Singh, 2018).

The structural classification of alkaloids further refines the understanding of their diversity. Alkaloids derived from tryptophan are referred to as indole alkaloids, and they comprise vinblastine and reserpine, which are known for their bioactivity in hypertension treatment and cancer therapy (Bhatla & Lal, 2018). Alkaloids that originate from tyrosine are known as isoquinoline alkaloids, and they include berberine and morphine, with potent antimicrobial and analgesic properties. Alkaloids that originate from or are synthesized from ornithine are called pyrrolizidine alkaloids. They are often associated with hepatotoxicity and occur in species of *Senecio* (Bhambhani *et al.*, 2021). Tropane alkaloids like atropine and cocaine exert profound effects on the nervous system. Quinoline alkaloids, notably quinine, play a pivotal role in treating malaria. In contrast, steroidal and pyridine alkaloids, including solanine and nicotine, exhibit potent bioactivity with applications ranging from pest control to stimulant consumption (Bhambhani *et al.*, 2021).

The ecological role of alkaloids in plant defense systems against insects and herbivores is exemplified by nicotine and caffeine. Nicotine disrupts the nervous systems of insects and herbivores, while caffeine interferes with insect feeding and metabolism (Roberts, 2013). Furthermore, alkaloids also exhibit antimicrobial properties (for example, berberine, an isoquinoline alkaloid with antibacterial properties) and contribute to plant resistance against pathogens (Roberts, 2013). Some alkaloids also function as allelopathic compounds, suppressing the growth of competing plants within their vicinity (for example, colchicine interferes with the cell division of neighbouring plant species). Additionally, some alkaloids play a role in pollination by modulating nectar composition and thereby enhancing pollination efficiency (Roberts, 2013).

Beyond their ecological roles, alkaloids have found extensive applications in industries. Their significance in the pharmaceutical industry cannot be overemphasized, as alkaloids have been used as essential drugs. Morphine remains one of the most effective analgesics, while quinine remains a frontline treatment for malaria (Salminen, 2018). Alkaloidal compounds like vincristine and vinblastine from *Catharanthus roseus* have revolutionized chemotherapy and cancer research due to their anticancer properties. Ephedrine has been widely used in respiratory therapies due to its bronchodilatory effects (Bhatla & Lal, 2018). In agriculture, as aforementioned, nicotine-derived compounds are used as alkaloid-based insecticides with proven efficacy in pest management, while alkaloids like cucurbitacins deter herbivorous insects (Salminen, 2018). The food and beverage industries have capitalized on the alkaloids caffeine and theobromine, integral components of the widely consumed stimulants, coffee, tea, and chocolate. Furthermore, the field of biotechnology has witnessed a growing interest in harnessing alkaloid biosynthetic pathways in microbial systems for scalable and sustainable production (Gardea *et al.*, 2017).

Regardless of their enormous potential, alkaloid research presents notable challenges as their structural complexities often hinder large-scale chemical synthesis and complicate their analysis. Furthermore, the inherent toxicity of certain alkaloids limits their applicability in medicine and agriculture, necessitating precise dosage control and regulatory oversight (Roberts, 2013). However, significant opportunities exist in metabolic engineering and synthetic biology as advances in genome sequencing, heterologous expression systems, and metabolic pathway reconstruction have paved the way for the enhanced production of alkaloids in microbial hosts, thereby offering sustainable alternatives to traditional plant-based extraction (Gardea *et al.*, 2017). The discovery of novel alkaloids with unique bioactivities also holds promise for developing next-generation pharmaceuticals, thereby cementing their legacy as an indispensable class of secondary metabolites with profound ecological, medicinal, and industrial significance.

1.3 Lignin

Lignin, a complex and highly branched phenolic polymer, ranks as the second most abundant biopolymer on Earth, following cellulose, and constitutes approximately 30% of the organic carbon in the biosphere (Boerjan *et al.*, 2003). It is a product of secondary metabolism

that serves as a structural element, maintaining the integrity of the plant cell wall. It provides rigidity and strength to vascular plants, facilitating the transport of water and solutes through the tracheary elements in the vascular system (Boerjan *et al.*, 2003). Lignin plays a broad role in plant growth, tissue and organ development, load-bearing capacity, and tolerance to biotic and abiotic stresses. Lignification serves as a protective mechanism against pathogens and herbivores by rendering plant tissues physically tough, making them indigestible to insects and other herbivores (Jan *et al.*, 2021). The process of plant lignification, occurring within the cell wall, involves three stages: the biosynthesis of monolignols in the cytosol, the transport of these monolignols to the cell wall, and their subsequent oxidative dehydrogenation and polymerization to form heterogeneous macromolecules (Liu, 2012).

The amorphous heteropolymeric lignin is primarily comprised of aromatic monolignols, including coniferyl alcohol, p-coumaryl alcohol, and sinapyl alcohol. These monolignols form, respectively, the guaiacyl (G-lignin), p-hydroxyphenyl (H-lignin), and syringyl (SH-lignin) units in lignin (McKenna *et al.*, 2013; Kamimura *et al.*, 2017; Kamimura *et al.*, 2019). Each unit is interconnected through C–O–C ether and C–C bonds, which result from the oxidative coupling of monolignols. The most prevalent linkage is the β -O-4 (β -aryl ether) bond, although lignin also contains other linkages, including β -5 (phenylcoumaran), 5-5 (biphenyl), β - β (pinoresinol), and β -1 (diarylpropane) bonds (Kamimura *et al.*, 2017).

Lignin, cellulose and hemicellulose constitute the primary components of lignocellulosic biomass within the plant cell wall (Lu *et al.*, 2017). Lignocellulosic biomass is the most abundant renewable resource in nature, with approximately 100 million tons of lignin produced globally, corresponding to a total value of approximately \$732.7 million (Bajwa *et al.*, 2019). Lignin accounts for 15-35% by weight of lignocellulosic biomass and represents the most plentiful source of renewable aromatic molecules in the biosphere (Weng *et al.*, 2021). Due to its high carbon-to-oxygen ratio and rich aromatic structure, lignin holds promise as a renewable feedstock for energy supplementation and a substrate for producing aromatic chemicals (Weng *et al.*, 2021). Aromatic compounds derived from plants and lignin have significant industrial and commercial uses. These include applications in the synthesis of plastics, biochemical building blocks for biorefineries, cosmetics, pharmaceuticals and food additives like preservatives, flavouring agents, and

antioxidants (McKenna *et al.*, 2013; Tsuge *et al.*, 2016). With the growing demand for plastics each year, plastics derived from renewable aromatic compounds offer a sustainable solution to meet this increasing need.

1.4 Catabolism of aromatic compounds

Lignin biodegradation involves the breakdown of native lignin to produce low molecular weight aromatic compounds, followed by mineralization (De-Gonzalo *et al.*, 2016). This biodegradation is an oxidative process that requires extracellular ligninolytic enzymes like lignin peroxidase, manganese-dependent peroxidase, versatile peroxidase, dye-decolorizing peroxidase, and laccases (Yadav *et al.*, 2022). These enzymes catabolize lignin to produce valuable chemicals like vanillin and vanillic acid that are used in flavourings and perfumes (Jiang *et al.*, 2023). Certain bacteria and fungi secrete a plethora of enzymes capable of catalyzing oxidative and hydroxylation reactions involved in lignin degradation (Zeng *et al.*, 2014). With ongoing research, an increasing array of lignin-degrading enzymes originating from both fungi and bacteria has been identified and employed in lignin depolymerization and mineralization processes. The type and structure of lignin influence the binding affinity of the lignin-degrading enzymes.

In fungi, the primary extracellular enzymes secreted by white-rot fungi for lignin degradation are oxidases and peroxidases. These enzymes catalyze oxidative reactions that involve cleaving carbon-carbon bonds and ether linkages and removing side chains and aromatic rings from lignin (Zabel & Morrell, 2020). Bacteria that exhibit lignin degradation capabilities are found in environments like soil, decaying wood, wastewater treatment plants, and animal intestines (Xu *et al.*, 2018). While bacteria may not match the lignin degradation efficiency of fungi, they exhibit greater adaptability to diverse environmental conditions. Research indicates that Actinobacteria, Proteobacteria, and Firmicutes are prominent lignin-degrading bacterial groups (Bugg *et al.*, 2011). These microbes are widely distributed across natural ecosystems, thriving in terrestrial and aquatic habitats (Srinivasan *et al.*, 2023).

The degradation of lignin by microbes produces a diverse mixture of aromatic compounds that serve as carbon and energy sources for fungi and bacteria. Almost all aromatic compounds obtained from plants that can be used as a source of carbon in fungi and aerobic bacteria are

funnelled into one of the seven aromatic intermediates, specifically catechol, protocatechuic acid, gentisic acid, hydroxyquinol, gallic acid, pyrogallol and hydroquinone, that further undergo ring cleavage and are subsequently converted to citric acid cycle intermediates (Lubbers *et al.*, 2019). The catabolic pathways of lignin-derived aromatic compounds have been studied in microbes, primarily in bacteria (Fuchs *et al.*, 2011) and, to a lesser extent, in fungi (Yaguchi *et al.*, 2017).

The syringyl (S), guaiacyl (G), and hydroxyphenyl (H) structural monomeric units produced during lignin depolymerization act as the starting points, progressing through upstream pathways to generate central aromatic intermediates such as catechol, protocatechuic acid, and 3-*O*-methyl gallic acid (Gu *et al.*, 2024). In bacteria, a diverse range of lignin-derived aromatic compounds with syringyl and guaiacyl nuclei are typically directed, through specific enzymatic reactions, into syringic acid and vanillic acid, respectively (Masai *et al.*, 2007). Syringic acid and vanillic acid are then converted to gallic acid and protocatechuic acid, aromatic compounds with catecholic structures, through an *O*-demethylation reaction (Chen *et al.*, 2012). A methyl moiety is transferred to tetrahydrofolate to form 5-methyl-tetrahydrofolate, which is used for C₁ metabolism and methionine synthesis in the *O*-demethylation reaction. Gallate and protocatechuate are further metabolized through the ring-cleavage pathways, and the metabolites produced are subsequently integrated into the tricarboxylic acid (TCA) cycle (Nogales *et al.*, 2011).

Studies on the metabolism of aromatic compounds by fungi have been reviewed (Makela *et al.*, 2015). Beginning with the structural polymeric molecule (lignin), microbial degradation, specifically by the white-rot fungus *Phanerochaete chrysosporium*, releases vanillin (Ahmad *et al.*, 2022). The vanillin, which is released, can be oxidized by the yeasts *Trichosporon oleaginosus* and *Debaryomyces hansenii*, as well as the filamentous fungus *Aspergillus japonicus*, to vanillic acid (Mathew *et al.*, 2007; Yaguchi *et al.*, 2017). An alternative metabolic pathway that converts vanillin to vanillyl alcohol has been reported to occur in the filamentous fungi, *A. japonicus*, *Penicillium simplicissimum*, and *Schizophyllum commune*, and in the yeast, *D. hansenii* (de Jong *et al.*, 1992; Mathew *et al.*, 2007; Tsujiyama & Ueno, 2008).

The biodegradation of lignin by the fungus *Phanerochaete chrysosporium* yields both vanillic acid and isovanillic acid (Ahmad *et al.*, 2022). The biotransformation of vanillic acid to protocatechuic acid through a demethylation reaction has been reported in the filamentous fungi *S. commune* and *A. japonicus* (Tsujiyama & Ueno, 2008; Rosini *et al.*, 2023). The conversion of vanillic acid, through a non-oxidative decarboxylation reaction, to guaiacol has also been observed in several fungi, including *Aspergillus* species (Guiraud *et al.*, 1992). In addition, *P. chrysosporium* and *A. niger* have been reported to convert vanillic acid, via an oxidative decarboxylation reaction, to methoxy hydroquinone, which is further converted to hydroxyquinol (Guiraud *et al.*, 1992; Baqueiro-Pena *et al.*, 2010).

Another metabolic pathway for the conversion of vanillic acid in filamentous fungi, including *Trichoderma reesei*, is the production of vanillin from vanillic acid via reduction (Guiraud *et al.*, 1992; Lubbers *et al.*, 2021). In fungi, the enzymes which catalyze these reactions have not been completely characterized. Similarly, coniferyl alcohol, one of the three aromatic monolignols that serve as structural monomers of lignin, follows a pathway not fully understood in fungi. While the specific catabolic pathway for coniferyl alcohol has been demonstrated in bacteria, where it is converted to 5-hydroxyconiferylaldehyde and ferulic acid (Lubbers *et al.*, 2019), this pathway has not yet been established in fungi. However, in the fungi *Pycnoporus cinnabarinus* and *Trametes* sp., it was reported that ferulic acid was converted to coniferyl alcohol and coniferyl aldehyde (Patil & Yadav, 2018; Lubbers *et al.*, 2019).

The direct conversion of coniferyl aldehyde to ferulic acid has also been observed in the yeast *Saccharomyces cerevisiae*, but the enzymes catalyzing these reactions remain uncharacterized (Adeboye *et al.*, 2015). Similarly, vanillyl-alcohol oxidase, an enzyme found in the ascomycete *Penicillium simplicissimum*, catalyzes the hydroxylation reaction that converts eugenol to coniferyl alcohol (van den Heuvel *et al.*, 2004). The pathway for the conversion of aromatic eugenol to *p*-vinyl guaiacol has been described in the ascomycetes *Fusarium solani* and *Paecilomyces variotii*, as well as in the basidiomycete *Schizophyllum commune* (Ghosh *et al.*, 2005; Lubbers *et al.*, 2019). The enzymes responsible for catalyzing this reaction in both bacteria and fungi have not yet been fully characterized, highlighting a recurring gap in our understanding of lignin-related metabolic pathways in microbes. This gap is reflected in the incomplete

characterization of enzymes involved in the conversion of vanillic acid in fungi, further underscoring the need for more research into fungal metabolic pathways.

The degradation of monolignol coniferyl alcohol, a component of lignin, by *Bacillus* sp., *Rhodococcus jostii*, and *Pseudomonas putida* releases another plant aromatic compound, ferulic acid (Raj *et al.*, 2007; Bugg *et al.*, 2011). In addition to its presence in lignin, ferulic acid is also found in polysaccharides like pectin and xylan, where it forms crosslinks between them and with lignin via ferulic acid dimerization (Dilokpimol *et al.*, 2016). In bacteria, the biotransformation of ferulic acid to vanillin has been characterized in detail in *Bacillus subtilis*, *Amycolatopsis* sp., *Pseudomonas fluorescens*, *R. jostii*, and *P. putida* (Chen *et al.*, 2012; Graf *et al.*, 2016). In species like *Sphingobium* sp. strain SYK-6 and *Streptomyces* sp. strain V-1, the enzymes feruloyl-CoA synthase (also called ferulate-CoA ligase) or p-hydroxycinnamoyl-CoA synthetase catalyze the transformation of ferulic acid to feruloyl-CoA, which undergoes further conversion to vanillin through a non- β -oxidative decarboxylation pathway involving enoyl-CoA hydratase/aldolase (Masai *et al.*, 2007; Yang *et al.*, 2013). This pathway has also been reported in the yeasts *Rhodotorula rubra* and *Debaryomyces hansenii* (Huang *et al.*, 1993; Mathew *et al.*, 2007), with recent evidence also pointing to *Aspergillus luchuensis* (Taira *et al.*, 2018). An alternative route for ferulic acid metabolism in the ascomycete *Myceliophthora thermophila* involves its non-oxidative decarboxylation to p-vinyl guaiacol, which is subsequently converted to vanillic acid (Topakas *et al.*, 2003).

1.4.1 The seven central aromatic intermediates

Figure 1.1 shows the conversion of lignin-derived aromatic compounds into seven central intermediates (the yellow portion of Figure 1.1), prior to ring fission and conversion to TCA cycle intermediates, before finally being mineralized. These catabolic pathways are broadly conserved in fungi and bacteria, with notable differences (Vaillancourt *et al.*, 2006; Lubbers *et al.*, 2019).

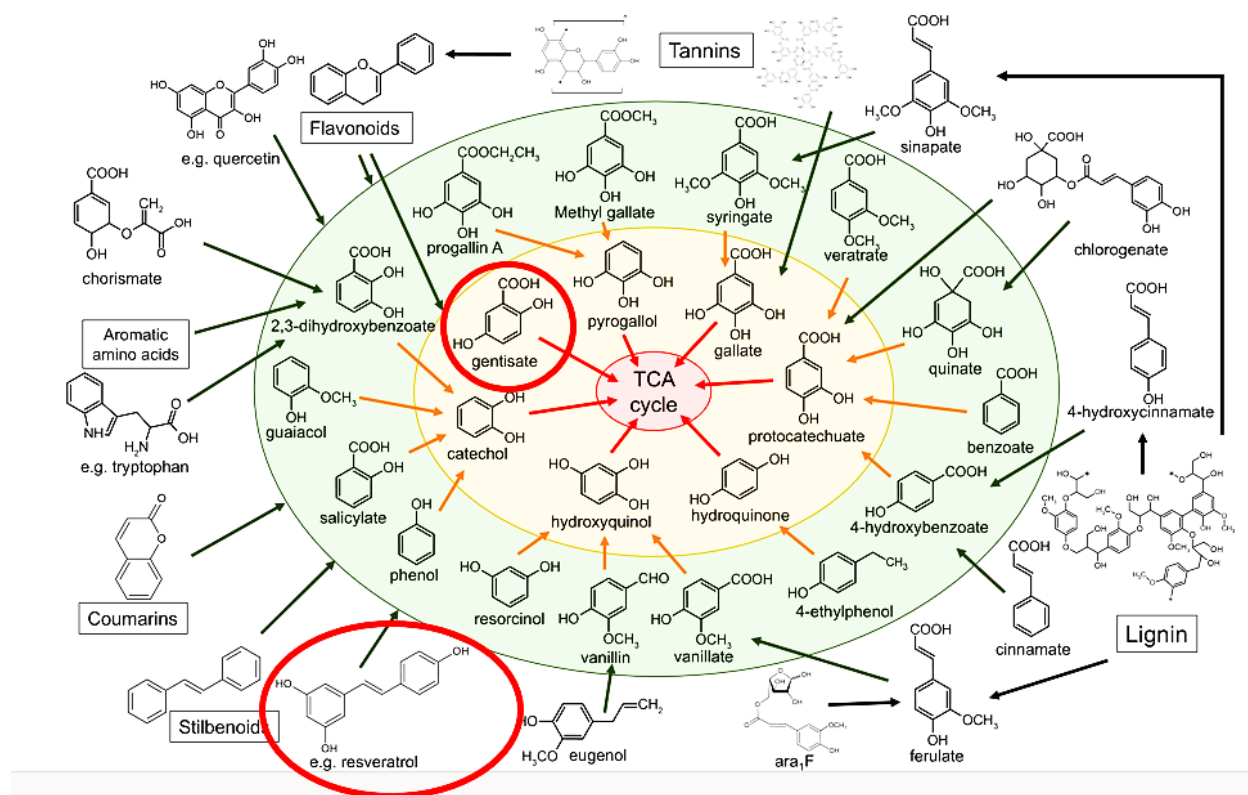


Figure 1.1 Plant aromatic compounds funnelled into seven central metabolic pathways (Modified from Phale et al. (2020)). The circled aromatic compounds are the foci of this study

The microbial degradation of aromatic compounds relies on ring cleavage pathways. These pathways play a pivotal role in detoxification and enable microorganisms to utilize aromatic compounds as a carbon source. Following ring cleavage, the resulting compounds undergo a series of transformations, ultimately yielding intermediates, fumarate, succinate, pyruvate, acetyl-CoA, and oxaloacetate that are integrated into the TCA cycle. It is noteworthy that ring cleavage pathways exhibit a high degree of conservation across different microorganisms, and in fungi, intradiol cleavage is the only observed mechanism (Semana & Powlowski, 2019).

1.4.2 Convergence of the catechol, protocatechuate, and hydroxyquinol branches into the β -Ketoadipate pathway

Figure 1.2 shows that the β -ketoadipate pathway serves as a common catabolic route for three aromatic intermediates: the catechol, protocatechuate, and hydroxyquinol pathways

(Harwood & Parales, 1996; MacLean *et al.*, 2006; Lubbers *et al.*, 2019). These branches arise from distinct precursors but ultimately converge at a common point, β -ketoadipate enol-lactone, which is then converted into TCA cycle intermediates (Michielse *et al.*, 2012). While bacteria and fungi utilize these pathways, their enzymatic mechanisms and cleavage strategies exhibit notable differences. For example, the catabolism of 3,4-dihydroxybenzoic acid (protocatechuic acid) in the filamentous fungus *A. niger* involves the conversion of 3-carboxy-*cis*, *cis*-muconic acid to 3-carboxymuconolactone, whereas the bacterial pathway yields 4-carboxymuconolactone (Kajander *et al.*, 2002; Becker *et al.*, 2018; Sgro *et al.*, 2023). Furthermore, in the conversion of carboxymuconolactone to β -ketoadipate, the fungal pathway employs a single enzyme possessing both decarboxylase and hydrolase activities, whereas the characterized bacterial pathway involves two enzymes (Ornston, 1966; Cain *et al.*, 1968; Sgro *et al.*, 2023).

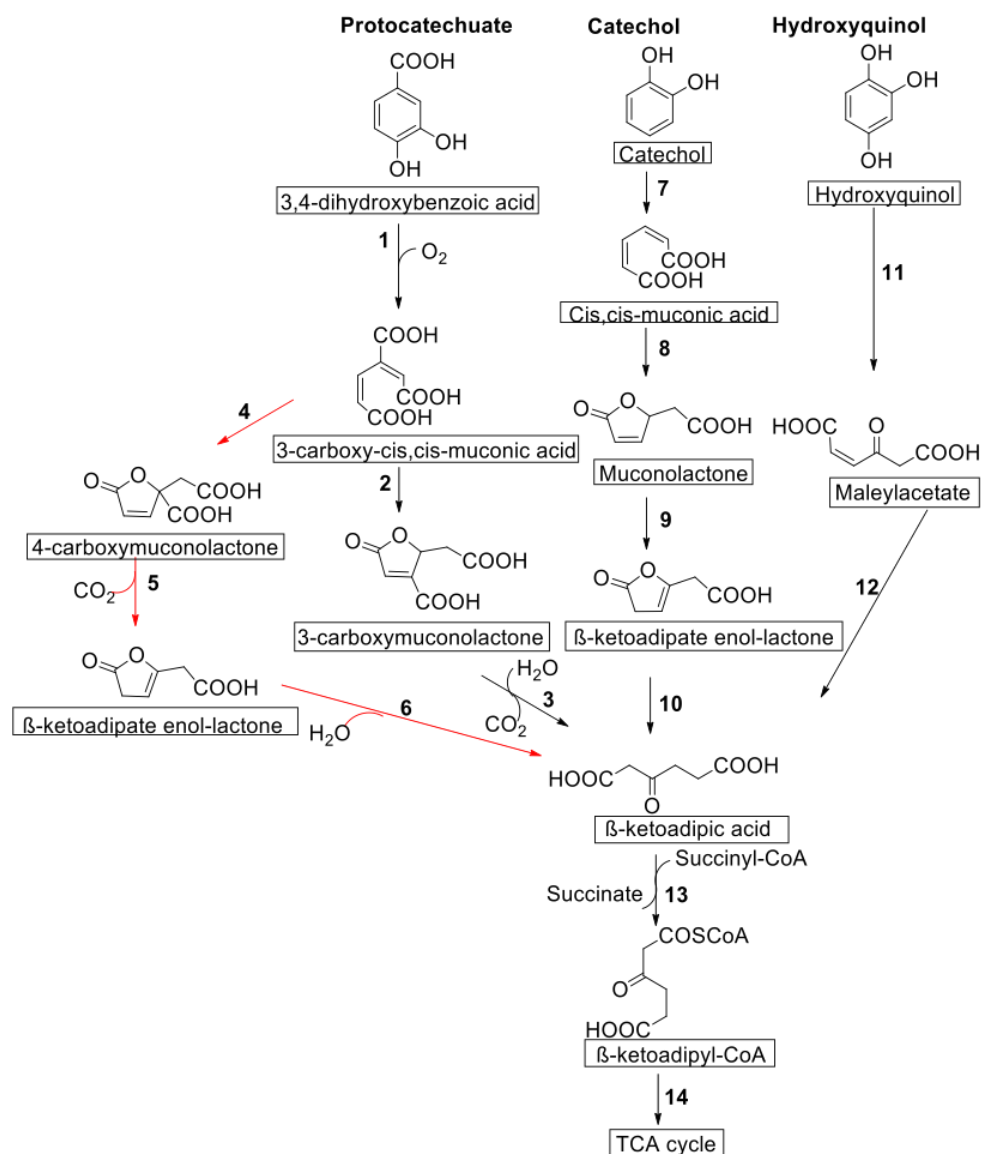


Figure 1.2 The bacterial and fungal metabolic pathways for protocatechuate, catechol, and hydroxyquinol converge into the β -ketoadipate Pathway. Red arrows indicate bacterial-specific steps. Fungi differ by converting 3-carboxy-cis, cis-muconic acid to 3-carboxymuconolactone, then directly to β -ketoadipate using a bifunctional 3-carboxymuconolactone hydrolase/decarboxylase. The Numbers in the figure represent enzyme for each step and are shown as, (1) protocatechuate 3,4-dioxygenase; (2) 3-carboxy-cis, cis-muconate cyclase; (3) 3-carboxymuconolactone hydrolase/decarboxylase, (4) 3-carboxy-cis,cis-muconate cycloisomerase, (5) 4-carboxymuconolactone decarboxylase, (6) β -ketoadipate enol-lactone hydrolase, (7)

catechol 1,2-dioxygenase, (8) muconate cycloisomerase, (9) muconolactone isomerase, (10) β -keto adipate enol-lactone hydrolase, (11) hydroxyquinol dioxygenase, (12) maleylacetate reductase, (13) β -keto adipate: succinyl-CoA transferase, and (14) β -keto adipyl-CoA thiolase.

1.4.3 Hydroquinone degradation pathway in bacteria and fungi

Fungi and bacteria employ different strategies for the catabolism of hydroquinone (Lam *et al.*, 2012; Lubbers *et al.*, 2019). In bacteria, hydroquinone degradation follows an estradiol cleavage pathway catalyzed by the enzyme chlorohydroquinone 1,2-dioxygenase, which has been identified in *Pseudomonas putida*, *P. fluorescens* ACB, and *Sphingobium* sp. strain SYK-6, that converts hydroquinone into 4-hydroxymuconic semialdehyde (Miyauchi *et al.*, 1999; Arora & Jain, 2012; Lubbers *et al.*, 2019; Spence *et al.*, 2020). The second step converts 4-hydroxymuconic semialdehyde into maleylacetate (Miyauchi *et al.*, 1999) and subsequently processes maleylacetate into 3-oxoadipate, which is then integrated into the TCA cycle for complete cellular assimilation (Miyauchi *et al.*, 1999; Lubbers *et al.*, 2019). This bacterial degradation route corresponds to the lower branch in Figure 1.3, where hydroquinone is converted into 4-hydroxymuconic semialdehyde (via enzyme III), then to maleylacetate (via enzyme IV), and subsequently enters the β -keto adipate pathway (via enzyme V).

Unlike bacteria, the strategy employed by fungi for the catabolism of hydroquinone is different. Fungi prioritize hydroxylation over direct cleavage to mitigate cytotoxicity, and this protective mechanism involves the enzymatic conversion of hydroquinone to hydroxyquinol (1,2,4-benzenetriol) by hydroquinone hydroxylase, an enzyme identified in the yeast *Candida parapsilosis* (Eppink *et al.*, 1997; Eppink *et al.*, 2000; Lubbers *et al.*, 2019). Hydroxyquinol then undergoes oxidative cleavage via hydroxyquinol 1,2-dioxygenase, yielding maleylacetate. Maleylacetate is subsequently reduced by maleylacetate reductase in an NADH-dependent reaction, forming 3-oxoadipate (Eppink *et al.*, 1997). This metabolite is further funnelled into the β -keto adipate pathway, where it is sequentially converted into β -keto adipate enol-lactone, hydrolyzed to β -keto adipate, and ultimately transformed into succinyl-CoA and acetyl-CoA (Eppink *et al.*, 1997; Eppink *et al.*, 2000; Lubbers *et al.*, 2019). These intermediates integrate into the TCA cycle, facilitating complete carbon assimilation (Eppink *et al.*, 1997; Eppink *et al.*, 2000; Lubbers *et al.*, 2019). While bacteria employ estradiol cleavage to directly break down

hydroquinone, fungi first convert it into hydroxyquinol before undergoing ring cleavage. These differences reflect evolutionary adaptations to manage cytotoxic intermediates and optimize metabolic efficiency. The fungal pathway is represented by the upper branch in Figure 1.3, where hydroquinone is first hydroxylated to 1,2,4-benzene-triol (via enzyme I), then cleaved to maleylacetate (via enzyme II), and finally channelled into the β -ketoadipate pathway through the action of enzyme V.

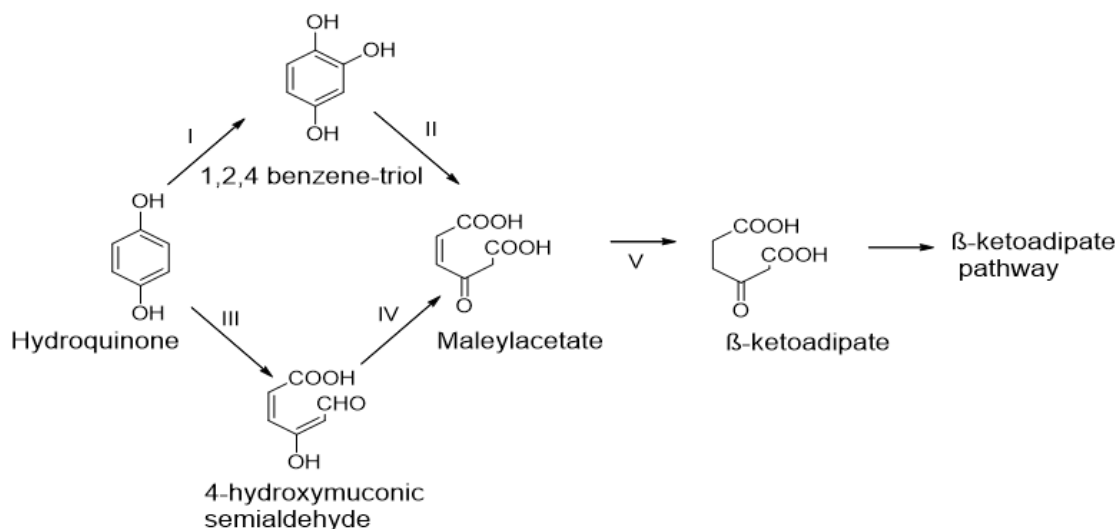


Figure 1.3 Pathways for the biodegradation of hydroquinone under aerobic conditions. The enzymes involved are I, hydroquinone hydroxylase; II, 1,2,4-trihydroxybenzene 1,2-dioxygenase; III, hydroquinone dioxygenase; IV, 4-hydroxymuconic semialdehyde dehydrogenase; and V, β -ketoadipate oxidoreductase.

1.4.4 Catabolism of gallate in bacteria and fungi

Tannase is an enzyme that catalyzes the hydrolysis of tannins into gallic acid and glucose, a process observed in bacteria, *Lactobacillus plantarum* and *Pantoea agglomerans*, as well as in the fungi *A. niger* and *Penicillium* spp. Once liberated, gallic acid undergoes non-oxidative decarboxylation by gallic acid decarboxylase, yielding pyrogallol and CO₂ (Li *et al.*, 2006).

The microbial degradation of gallic acid follows distinct yet convergent pathways in bacteria and fungi. In bacteria, it was reported that gallic acid undergoes intradiol cleavage by gallate dioxygenase, yielding 4-oxalomesaconate (Kasai *et al.*, 2005; Müller *et al.*, 2007). This intermediate is further converted through a metabolic route analogous to the protocatechuic acid 4,5-cleavage pathway, ultimately generating pyruvate and oxaloacetate, which seamlessly integrate into the TCA cycle (Kasai *et al.*, 2005; Nogales *et al.*, 2011).

In fungi, gallate metabolism exhibits similarities and unique variations compared to bacterial metabolic pathways. The catabolism of gallate, particularly in Ascomycetes fungi, follows multiple pathways, although none have been fully elucidated (Martins *et al.*, 2024). One of the proposed pathways involves converting and reducing gallate into protocatechuate, as observed in *Penicillium simplicissimum* (Patel *et al.*, 1992). Another pathway involves the non-oxidative decarboxylation of gallate to pyrogallol, which subsequently undergoes intradiol cleavage via a catechol-like dioxygenase to form 2-hydroxymuconate, eventually entering a yet-undefined catabolic pathway for 4-oxalocrotonate as observed in *Blastobotrys adeninivorans* (Meier *et al.*, 2017). Similarly, *Aspergillus* and *Penicillium* species have been reported to accumulate pyrogallol, suggesting they follow a comparable pathway for degradation (Gurujeyalakshmi & Mahadevan, 1987). Additionally, *A. niger* and *A. flavus* have been proposed to perform direct ring cleavage of gallate, supported by evidence of gallate dioxygenase activity, and the catabolism of gallate in *A. niger* is independent of the protocatechuate branch of the 3-oxoadipate pathway (Martins *et al.*, 2024). These findings suggest that fungi exhibit diverse and possibly redundant metabolic strategies for gallate degradation.

1.4.5 Catabolism of pyrogallol in bacteria and fungi

In bacteria, pyrogallol metabolism serves as a metabolic route for degrading plant-derived polyphenols, particularly hydrolyzable tannins (Choi *et al.*, 2023). Degradation begins with the decarboxylation of gallic acid to pyrogallol, catalyzed by gallate decarboxylase, an enzymatic reaction observed in *Enterobacter*, *Citrobacter* and *Lactobacillus* (Yoshida & Yamada, 1985; Tabasco *et al.*, 2011; Li & Wang, 2015; Choi *et al.*, 2023). Once formed, pyrogallol undergoes extradiol ring cleavage mediated by pyrogallol dioxygenase, yielding 2-hydroxymuconic acid and 2-pyrone-6-carboxylic acid (Saeki *et al.*, 1980; Mayer & Que, 1984). This intermediate is then subsequently funnelled into metabolic pathways that converge with the TCA cycle (Saeki *et al.*, 1980; Mayer & Que, 1984). This pathway, illustrated in Figure 1.4, reflects the established degradation route used by the bacterium *Citrobacter freundii*. Regarding fungi, the metabolism of pyrogallol follows a parallel yet distinct pathway. In *Arxula adeninivorans*, pyrogallol is catabolized through an extradiol cleavage mechanism akin to bacterial pathways, leading to the formation of 2-hydroxymuconic acid (Boer *et al.*, 2009; Meier *et al.*, 2017). This pathway is distinct due to the involvement of a eukaryotic extradiol dioxygenase, which differs in enzyme structure, regulation, and metabolic context from its bacterial counterparts. Similarly, in *Aspergillus oryzae*, the pathway proceeds via pyrogallol dioxygenase, supporting the notion of a conserved fungal strategy for aromatic degradation (Wakai *et al.*, 2019).

In fungi and bacteria, gallic acid decarboxylase mediates the initial conversion of gallic acid to pyrogallol, thus providing an alternative entry point for gallic acid catabolism (Li *et al.*, 2006; Boer *et al.*, 2009; Meier *et al.*, 2017; Wakai *et al.*, 2019). However, the downstream degradation pathways diverge between these organisms. For instance, pyrogallol undergoes oxidative ring cleavage to form 2-hydroxymuconic acid. This process is catalyzed by pyrogallol 1,2 dioxygenase in *C. freundii*. The 2-hydroxymuconic acid formed is then subsequently funnelled into central metabolism through conversion into TCA cycle intermediates (Li *et al.*, 2006). In contrast, the degradation of gallic acid in fungi, as reported in *A. niger* (Arentshorst *et al.*, 2021), *Penicillium simplicissimum* (Hameed, 1987) and other species of fungi, often involves a more complex network of reactions. These fungi employ both extracellular oxidative enzymes, laccases, and intracellular dioxygenases (Makela *et al.*, 2014), but the sequence of reactions involved in the catabolism of gallic acid to central metabolites is more variable and less well characterized than in

bacteria. For example, some fungi have been shown to metabolize gallic acid via oxidative polymerization or transformation into other phenolic intermediates before ring cleavage occurs (Li *et al.*, 2006).

Although Figure 1.4 depicts the gallate degradation pathway in *C. freundii*, it was selected because it illustrates clearly the steps involved in breaking down gallate into TCA cycle intermediates. While fungal pathways converge on similar metabolic endpoints, pyruvate or fumarate, the enzymatic steps involved and their regulation differ markedly from the bacterial pathway. Hence, Figure 1.4 serves as a representative model to illustrate the core degradation strategy common to bacteria and fungi, emphasizing the bacterial system where enzymatic roles and intermediates have been established.

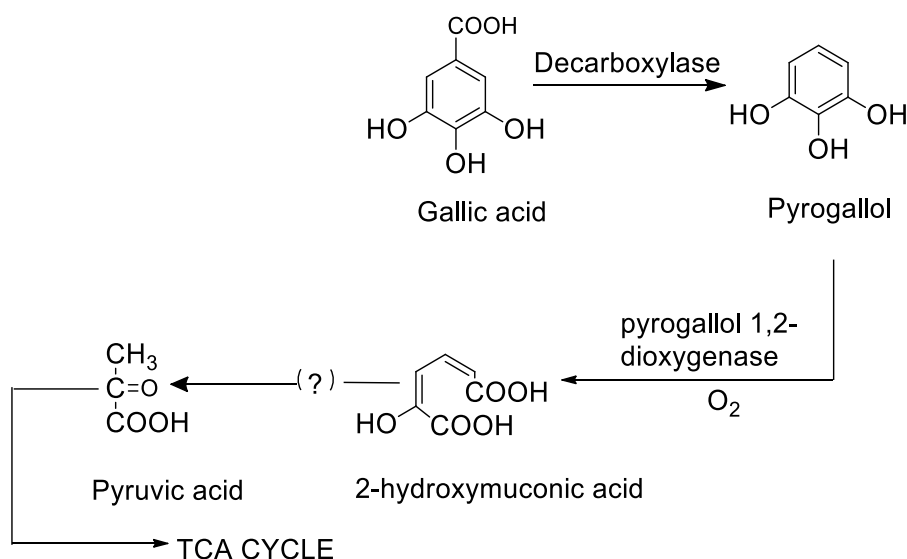


Figure 1.4 The proposed pathway for the degradation of gallotannins by *Citrobacter freundii*.

1.5 Gentisate and resveratrol

As shown in Figure 1.1, one of the seven central intermediates that emerge from these pathways is gentisate, which can be broken down further through ring fission and directed into the TCA cycle. Another compound that serves as a source of carbon and energy for microorganisms

is resveratrol. Stilbenoids (e.g, resveratrol) are catabolized through oxidation, reduction, hydroxylation and cleavage, which, like gentisate, are funnelled into the TCA cycle.

1.5.1 Gentisic acid (2,5-dihydroxybenzoic acid (2,5-DHB))

Gentisic acid (2,5-dihydroxybenzoic acid), or 2,5-DHB, is a diphenolic compound and a derivative of benzoic acid found and distributed in plant materials (Harris *et al.*, 2007), including derivatives of lignin and polycyclic aromatic hydrocarbons. In microbes, gentisic acid is a metabolic product of salicylic acid, *p*-hydroxybenzoic acid, and 3-hydroxybenzoic acid (Lubbers *et al.*, 2019).

In bacteria, gentisate catabolism is facilitated by the enzyme gentisate 1,2-dioxygenase, which requires oxygen and iron (II) as cofactors. This enzyme catalyzes the direct ring cleavage of gentisate to produce maleylpyruvate, which is subsequently metabolized through one of two alternative pathways that vary among bacterial species (Lubbers *et al.*, 2019). In one of the routes, it is broken down to produce pyruvate and maleate via the hydrolysis of maleylpyruvate by the enzyme maleylpyruvate hydrolase (Liu *et al.*, 2013). In another route, isomerization of maleylpyruvate occurs via the action of maleylpyruvate isomerase to yield fumarylpyruvate before it is hydrolyzed to pyruvate and fumarate (Romero-Silva *et al.*, 2013).

In bacteria, genes encoding the catabolic enzymes of the gentisate pathway are typically arranged in operons or clusters that are co-transcribed and induced by gentisate (Zhao *et al.*, 2004; Romero-Silva *et al.*, 2013). The operon organization enables the coordinated expression of all enzymes necessary for the conversion of gentisate to maleate or fumarate and pyruvate before they are channelled to the TCA cycle for complete mineralization (Zhao *et al.*, 2004; Xu *et al.*, 2012; Romero-Silva *et al.*, 2013). Aside from gentisate catabolic genes, specific genes that encode membrane transporters are also contained in the gentisate degradation operons, and they show specificity for aromatic compounds. For example, the *genK* gene in the *Corynebacterium glutamicum* gentisate degradation gene cluster codes for a gentisate-specific transporter (Xu *et al.*, 2012).

In some bacteria, gentisate catabolism gene clusters may include the aromatic ring hydroxylase enzymes, 3-hydroxybenzoate 6-hydroxylase, which converts 3-hydroxybenzoate to gentisate (Goetz & Harmuth, 1992; Romero-Silva *et al.*, 2013). The gentisate degradation pathway has been studied in *Ralstonia* species, where a 21.6 kb region containing the genes involved in the degradation of gentisate was sequenced, heterologously expressed, and biochemically characterized (Zhou *et al.*, 2001).

In fungi, based on sequence similarities to bacterial enzymes, candidates for enzymes involved in the degradation of aromatic compounds have been proposed (Vesth *et al.*, 2018; Aguilar-Pontes *et al.*, 2018). Experimental evidence for the functions of enzymes involved in gentisate catabolism in fungi has been limited to the proteomic and transcriptomic levels. Based on sequence similarity to bacterial enzymes and transcriptomic and proteomic profiling, gentisate catabolism in the yeast *Candida parapsilosis* was proposed to resemble the bacterial pathway involving fumarylpyruvate (Holesova *et al.*, 2011; Cillingova *et al.*, 2022). Neither the genes nor their encoded enzymes involved in the catabolism of gentisate were characterized.

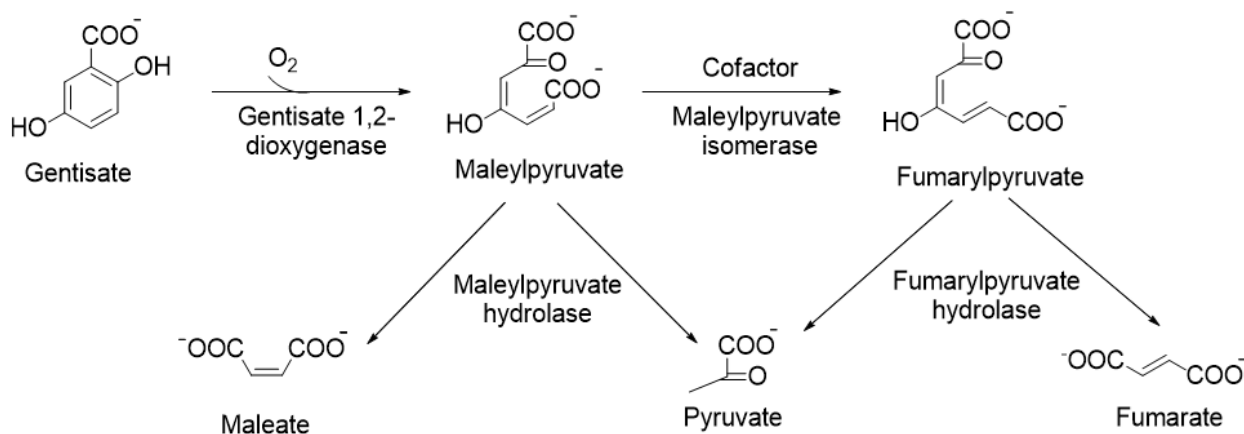


Figure 1.5 Catabolic pathway for gentisate conversion to pyruvate, maleate, and fumarate in bacteria. Maleylpyruvate isomerase activity is dependent on either glutathione, mycothiol, or L-cysteine as cofactors.

1.5.2 Resveratrol

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a member of a subclass of polyphenols characterized by two phenol rings connected by a styrene double bond. It is synthesized in plants through the phenylpropanoid pathway and is particularly predominant in the skin of grapes, berries, and peanuts (Feng *et al.*, 2022; Alesci *et al.*, 2022). Resveratrol exists in two isomeric forms: *trans*-resveratrol and *cis* resveratrol. The *trans*-form is more stable and exhibits biological activities that can benefit both plant and animal systems (Leischner *et al.*, 2021).

Similar to salicylates and their derivatives, resveratrol functions as a defense molecule in plant systems, protecting against pathogens, pests and environmental stress. The production of resveratrol is typically triggered as part of the innate immune response, especially under conditions of stress, pathogen attack, UV radiation, or physical damage (Song *et al.*, 2021; Yan *et al.*, 2021). Resveratrol acts as a phytoalexin, a substance produced by plants that inhibits the growth of invading microorganisms and whose biosynthesis is induced in response to exposure to pathogenic bacteria and fungi, as well as insect and herbivore-induced wounds (Jeandet *et al.*, 2019). Stilbenes originate from cinnamic acid, which is produced through the enzymatic activity of phenylalanine-ammonia lyase. Through a hydroxylation reaction catalyzed by cinnamate-4-hydroxylase, cinnamic acid is metabolized to *p*-coumaric acid, which is further converted by the enzyme *p*-coumaroyl: CoA ligase to *p*-coumaroyl-coenzyme A. Stilbene synthases catalyze the synthesis of resveratrol via the condensation of three molecules of malonyl-CoA with *p*-coumaroyl-coenzyme A, a process that involves four decarboxylation steps (Brefort *et al.*, 2011). Resveratrol has two geometric isomers, the *cis* and *trans* isomers. The *trans* isomer predominates and offers the most potent therapeutic benefits due to the reduced steric hindrance of its side chains (Cardile *et al.*, 2007). Exposure to heat, ultraviolet radiation and light can induce the isomerization of the *trans* isomer to the *cis* isomer (Camont *et al.*, 2009).

Resveratrol exhibits antimicrobial activity against both Gram-positive and Gram-negative bacteria, as well as antifungal activity (Fuloria *et al.*, 2022). For instance, resveratrol has been shown to inhibit the growth of bacterial pathogens associated with plants and animals, including foodborne pathogens (Zhang *et al.*, 2021; Hakalova *et al.*, 2022). Its mode of action against bacteria includes disrupting the bacterial cell wall and membrane, interfering with the function of

essential enzymes, and inhibiting nucleic acid synthesis, leading to impaired cell division and growth (Abedini *et al.*, 2021; Cebrian *et al.*, 2022).

Resveratrol inhibits the growth of the plant pathogenic fungus *Botrytis cinerea*, which causes grey mould in grapes and other fruits, as well as *Plasmopara viticola*, responsible for downy mildew in grapevines (Roca-Couso *et al.*, 2021; Wu *et al.*, 2023). The compound achieves this by disrupting the integrity of fungal cell walls and membranes, inhibiting spore germination, and impairing the synthesis of essential fungal components, such as ergosterol, a component of fungal cell membranes (Okamoto-Shibayama *et al.*, 2021). Moreover, resveratrol can interfere with the metabolic pathways that fungi use to regulate growth and reproduction. For example, it inhibits the activity of fungal enzymes, chitin synthase and glucan synthase, which are involved in cell wall synthesis (Adrian *et al.*, 1997). Additionally, resveratrol can induce the production of reactive oxygen species within fungal cells, leading to oxidative stress and cellular damage. The protective role of resveratrol extends beyond direct antimicrobial activity. It also strengthens plant cell walls by promoting the cross-linking of cell wall polymers, making it more difficult for pathogens to penetrate and establish infections. It can act as a signalling molecule, activating other defense mechanisms in plants, including the production of other phytoalexins, pathogenesis-related proteins, and the reinforcement of plant tissues through lignification (McCalley *et al.*, 2014; Jeandet *et al.*, 2020).

The catabolism of resveratrol by bacteria and fungi occurs through the action of lignostilbene- α , β -dioxygenases, also commonly referred to as stilbene cleavage dioxygenases. These iron-dependent enzymes, known for their broad substrate specificity, function by catalyzing the oxidative cleavage of the α , β -bond of resveratrol to yield smaller modified benzaldehydes, 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde. The stilbene cleavage dioxygenases are also part of a larger family of enzymes (best known for their oxidative cleavage of double bonds in larger compounds) called carotenoid cleavage oxygenases (Kamoda & Saburi, 1993; Kuatsjah *et al.*, 2021).

In the fungus *Ustilago maydis*, resveratrol is metabolized to two phenolic aldehydes, 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde, through the cleavage of its central C $_{\alpha}$ -C $_{\beta}$

double bond by a resveratrol cleavage oxygenase, an enzyme that specifically cleaves resveratrol (Brefort *et al.*, 2011). Similarly, both *Phomopsis liquidambari*, an endophytic fungus and *Acinetobacter oleivorans*, a rhizosphere bacterium, employ similar enzymes to cleave resveratrol. Specifically, resveratrol oxygenase in these organisms converts resveratrol into 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde, which are then metabolized to 4-hydroxybenzoic acid and 3,5-dihydroxybenzoic acid (Kurt *et al.*, 2018; Wang *et al.*, 2019). These compounds are subsequently funnelled into the TCA cycle.

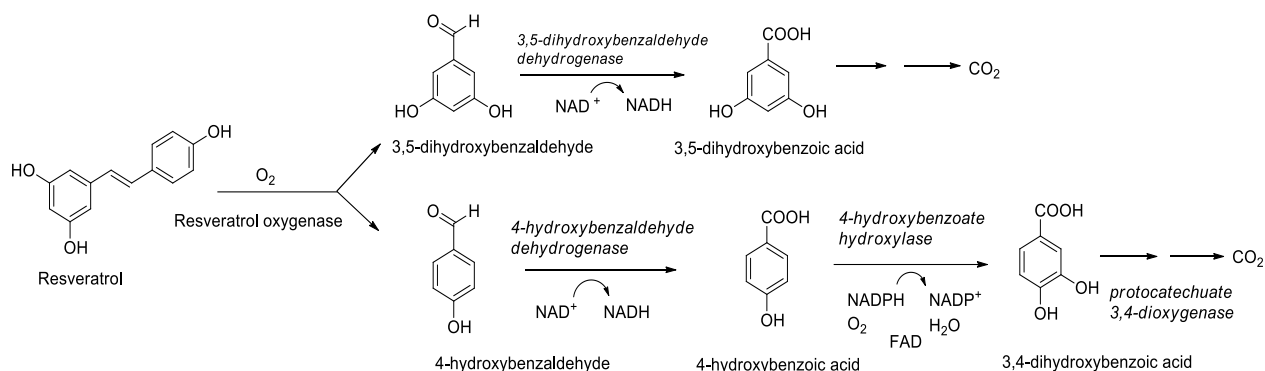


Figure 1.6 Proposed pathway for resveratrol degradation in fungi and bacteria

1.6 *Aspergillus niger* as a model organism

Aspergillus niger is a filamentous fungus belonging to the *Aspergillaceae* family (Yu *et al.*, 2021). This family encompasses over 300 species characterized by their distinctive conidiophores. *Aspergillus niger* is commonly found in soil and in decomposing plant matter. It is known for its black-coloured spores and ability to produce citric acid (Behera, 2020). It harbours a vast array of genes that regulate both primary and secondary metabolisms (Pel *et al.*, 2007). It also effectively metabolizes a variety of plant polysaccharides using an extensive array of extracellular enzymes (de Vries *et al.*, 2017). In addition, *A. niger* plays a significant role in industrial enzyme production, particularly in converting plant-based feedstocks into fermentable sugars (Andersen *et al.*, 2011). Globally, the fungus is utilized in the production of the majority of citric acid via fermentation (Tong *et al.*, 2019) as it has a tolerance to low pH, and it has an efficient protein secretion system (Kwon *et al.*, 2012; Cairns *et al.*, 2018).

Aspergillus niger stands out as one of the most studied organisms in filamentous fungal research. Its genetic manipulability, facilitated by tools, CRISPR-Cas9, makes it particularly advantageous (Song *et al.*, 2018). Moreover, *A. niger* boasts high-quality genome sequences (Pel *et al.*, 2007; Andersen *et al.*, 2011). The *A. niger* genome NRRL3 has been fully manually curated (Aguilar-Pontes *et al.*, 2018) and is thus a reference for genome-wide studies. *Aspergillus niger* strain CBS 138852 possesses mutations that are advantageous for genetic manipulation studies. It originates from strain NRRL3 with multiple mutations that facilitate its genetic manipulability, including *cspA1*, resulting in short, dense conidiophores for easy isolation on solid media (Bos *et al.*, 1988); *pyrG* (orotidine-5'-phosphate decarboxylase gene) providing bidirectional selection of gene transformants (Goosen *et al.*, 1987); and $\Delta kusA$ (gene involved in non-homologous end-joining DNA repair pathway) to promote homologous recombination. (Meyer *et al.*, 2014).

1.7 Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas)

First recognized as a defense strategy for bacteria against bacteriophages (Barrangou *et al.*, 2007), the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 system (CRISPR/Cas9) has revolutionized genomic manipulations by providing a precise, adaptable, and efficient tool for genome editing. This system has been harnessed for targeted genome modification across a wide range of organisms (Jiang *et al.*, 2013), including filamentous fungi like *A. niger* (Nodvig *et al.*, 2015; Song *et al.*, 2018). The core components of the Class 2 CRISPR/Cas system include the Cas9 endonuclease and the guide RNA (gRNA).

1. Cas9 endonuclease: This enzyme introduces double-stranded breaks in DNA at specific locations as directed by the gRNA. The ability of Cas9 to create precise cuts enables targeted genetic modifications, which can range from gene disruption to the insertion of new sequences. By leveraging cellular repair pathways, these breaks can lead to small mutations or specific alterations directed by repair templates.
2. Guide RNA (gRNA): Composed of a scaffold sequence and a 20-base pair protospacer sequence (crRNA), the gRNA is essential for directing Cas9 to the precise DNA location

to be edited. The protospacer sequence pairs with the target DNA, while the scaffold portion of the gRNA forms a complex with Cas9, guiding it to the intended target. The Protospacer Adjacent Motif (PAM), three nucleotides with the sequence NGG, must be adjacent to the target site for targeting. Once the crRNA matches the target DNA sequence and binds next to the PAM site, Cas9 induces the double-stranded break, enabling subsequent editing.

In addition to Cas9, other Class 2 nucleases such as Cas12a (Cpf1) have been used to effectively manipulate the genomes of fungi. In contrast to Cas9, Cas12a does not require a tracrRNA, but processes its own crRNA, and recognizes a T-rich PAM (5'-TTN/TTTN-3') instead of the G-rich PAM required by Cas9. Cas12a generates staggered double-stranded breaks with 5' overhangs, which can facilitate certain types of DNA insertions. The PAM requirement of Cas 12a is less rigorous, thus expanding the number of accessible genomic targets. Its versatility as a complement to Cas9 in fungal research has been demonstrated by its successful application in different species of fungi, such as *Yarrowia* and *Aspergillus* (Ouedraogo & Tsang, 2020).

A typical CRISPR-Cas system workflow for genome editing (Figure 1.7) begins with designing a specific guide RNA (gRNA) to target a gene of interest, and then delivering both the Cas protein and the gRNA into cells. Following double-stranded breaks by Cas protein (Cas9 or Cas12a), cellular repair mechanisms (either non-homologous end joining or homology-directed repair) complete the process, resulting in gene knockouts, insertions, or other modifications (Ouedraogo & Tsang, 2020).

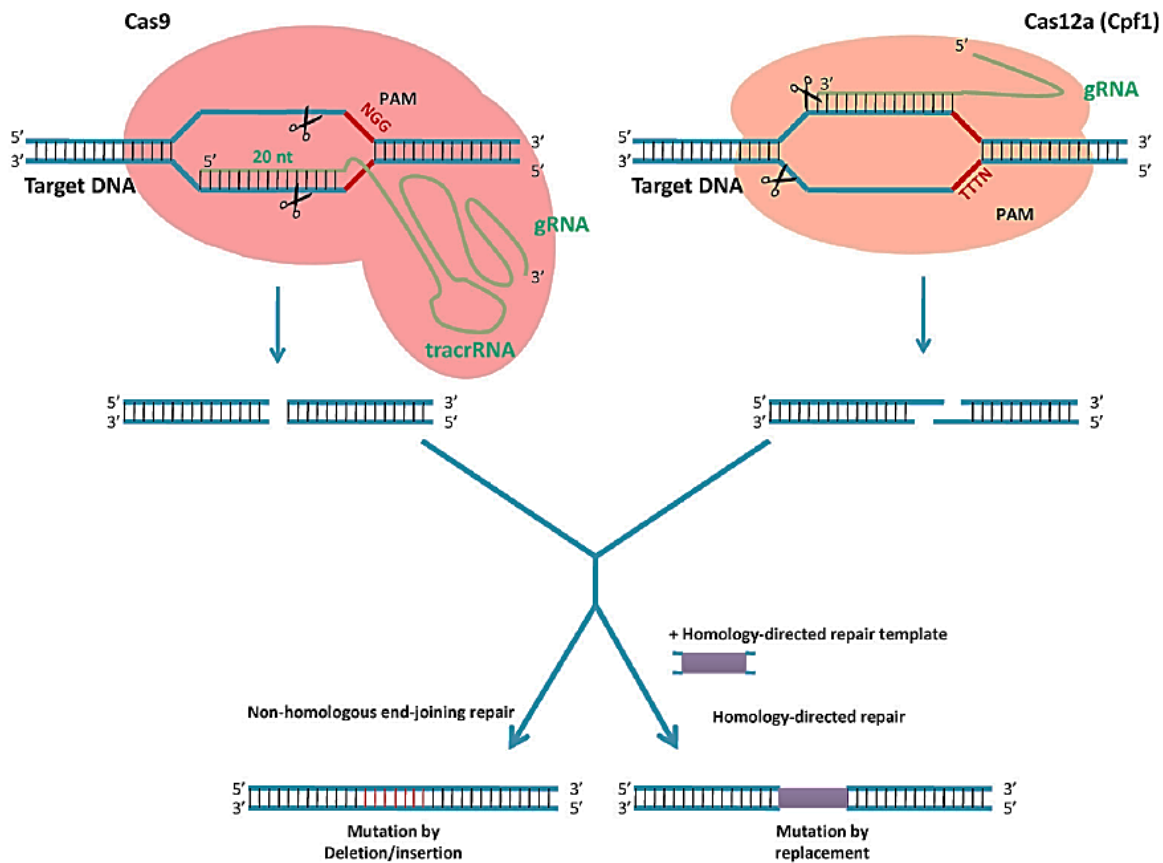


Figure 1.7 Genome editing mediated by Cas9 and Cas12a CRISPR-Cas systems
(Source: (Ouedraogo & Tsang, 2020))

1.8 Rationale for the study

Lignin is the second most abundant biopolymer on the planet. It is rich in aromatic compounds, which can serve as precursors for industrially relevant chemicals (Bajwa *et al.*, 2019). However, its complex and heterogeneous structure presents challenges in its breakdown and valorization (Adeboye *et al.*, 2015).

The breakdown or degradation of lignin produces a variety of aromatic compounds, many of which are toxic to living organisms. This toxicity poses a significant challenge in bioprocessing, for example, in fermentation, where plant biomass is used as a feedstock (Lubbers *et al.*, 2019; Jiang *et al.*, 2024). Additionally, toxic mono- and polycyclic aromatic pollutants, benzene, toluene,

ethylbenzene, and xylene, are commonly found in petroleum-contaminated environments and pose severe threats to human health due to their carcinogenic, mutagenic and teratogenic effects (Bulatovic *et al.*, 2022). Nevertheless, certain fungi and bacteria have developed enzymatic machinery to degrade these compounds, thereby contributing to their mineralization into less toxic forms and the production of valuable bioproducts.

Many of the intermediates of aromatic degradation hold industrial value and can serve as precursors for the synthesis of bio-based chemicals. For example, *cis*, *cis*-muconic acid is an intermediate in the production of adipic acid, a crucial component in the manufacture of nylon and polymers (Vardon *et al.*, 2015). Other valuable intermediates include pyridine 2,4-dicarboxylic acid, 2-pyrone-4,6-dicarboxylic acid, pyridine 2,5-dicarboxylic acid, 2-hydroxymuconic semialdehyde, 3-carboxymuconate, and muconolactone (Beckham *et al.*, 2016). Understanding the pathways involved in aromatic compound degradation will help redirect lignin-derived intermediates into commercially valuable bioproducts.

Multiple pathways funnel degraded aromatic compounds into central metabolism, primarily through seven core intermediates: catechol, protocatechuate, gentisate, hydroxyquinol, hydroquinone, gallate, and pyrogallol (Lubbers *et al.*, 2019). Among these, gentisic acid is an essential metabolic product of the plant-based aromatics salicylic acid, p-hydroxybenzoic acid, and 3-hydroxybenzoic acid (Lubbers *et al.*, 2019). The study of these pathways provides insights into microbial degradation of complex aromatic compounds.

Despite progress in understanding bacterial degradation pathways of aromatics, substantial gaps remain in their fungal metabolism. These knowledge gaps exist in parts of the gentisate pathway and pathways involving resveratrol and other central intermediates of catabolized aromatic compounds funnelled into the TCA cycle. *Aspergillus niger* is known for its metabolic versatility in degrading toxic aromatic compounds. It has been reported to degrade chlorinated aromatic compounds via the β -ketoadipate pathway and can break down other environmental pollutants (Lubbers *et al.*, 2019; Sgro *et al.*, 2023). Given its diverse catabolic potential and its established role as an industrial cell factory, *A. niger* is a promising candidate for further exploration and valorization of aromatic degradation mechanisms.

The advantages of *A. niger* include its well-annotated genome and genetic tractability. The availability of high-quality genomic resources and powerful molecular tools like the CRISPR-Cas9 genome editing enables functional analysis of genes involved in aromatic degradation (Nodvig *et al.*, 2015; Song *et al.*, 2018; Jin *et al.*, 2022). These resources make *A. niger* an ideal model organism to unravel the metabolic circuitry of fungal degradation pathways and apply this knowledge to environmental and industrial applications.

1.9 Specific goal of the study

The primary aim of this research is to identify and characterize the genes catalyzing each step in the catabolic pathways of gentisate and resveratrol in *A. niger*. To achieve this, a multi-pronged approach was adopted. The first aspect involves a dual strategy for gene identification: comparative analysis through homology to functionally characterized genes encoding enzymes involved in gentisate and resveratrol catabolism in both bacterial and fungal species, and transcriptome profiling to detect genes that are differentially upregulated under distinct growth conditions. The second approach employs CRISPR-Cas9 technology to generate knockout mutants for the most promising candidate genes, followed, where warranted, by complementation studies to restore gene function in the mutant strains. In the third aspect, phenotypic analysis of these mutants, grown on gentisate or resveratrol as the sole carbon source, is conducted to determine the specific involvement of each gene in the respective catabolic pathways. Finally, metabolic profiling using liquid chromatography-mass spectrometry, with gentisate or resveratrol as substrates, is carried out to detect the accumulation of pathway intermediates in the pathway mutants to support the accurate functional assignment of each gene.

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Chapter 2: Functional characterization of gentisate catabolism in *Aspergillus niger* reveals a gene encoding a CENP-V/GFA domain-containing protein is responsible for the conversion of maleylpyruvate to fumarylpyruvate

2.1 Abstract

One of the pathways for the aerobic degradation of aromatic compounds is the gentisate (2,5-dihydroxybenzoic acid) pathway. In bacteria, the degradation of gentisate begins with ring cleavage oxidation by gentisate 1,2-dioxygenase, yielding maleylpyruvate. The fate of maleylpyruvate can proceed either via its isomerization to fumarylpyruvate with subsequent hydrolysis to fumarate and pyruvate, or maleylpyruvate can be directly hydrolyzed to yield maleate and pyruvate. Here we show that a cluster of five genes, *NRRL3_05233-NRRL3_05237*, was upregulated when *Aspergillus niger* was grown on gentisate. Strains carrying deletion mutations in four of the genes displayed a growth defect on gentisate, suggesting that they are responsible for gentisate catabolism. Based on analyses of protein domains and metabolites accumulated by the deletion mutants, we deduce that *NRRL3_05233* (*gtaR*) encodes the transcription regulator of the gentisate clustered genes, *NRRL3_05236* (*gdoA*) encodes the gentisate 1,2-dioxygenase in the conversion of gentisate to maleylpyruvate, and *NRRL3_05237* (*fphA*) encodes fumarylpyruvate hydrolase in the conversion of fumarylpyruvate to fumarate and pyruvate. Δ *NRRL3_05235* was unable to grow on gentisate and accumulated a metabolite with a molecular mass of maleylpyruvate. Growth of Δ *NRRL3_05235* on gentisate was restored by complementation with the gene encoding the bacterial glutathione-dependent maleylpyruvate isomerase or its *A. niger* orthologues, suggesting that Δ *NRRL3_05235* is involved in the conversion of maleylpyruvate to fumarylpyruvate. *NRRL3_05235* lacks sequence and structural similarity to known maleylpyruvate isomerases. *NRRL3_05235* contains a CENP-V/GFA (centromere protein V / glutathione-dependent formaldehyde activating enzyme) domain. The bacterial protein with this domain is a carrier of glutathione with no catalytic activity. For this reason, we propose that *NRRL3_05235* (*miaA*) encodes a maleylpyruvate isomerization-activating protein, possibly with no catalytic activity by itself.

2.2 Introduction

The breakdown of aromatic compounds by microorganisms is essential for carbon cycling, particularly in environments rich in plant-derived materials—lignin, tannins and their derivatives (Mäkelä *et al.*, 2015; Janusz *et al.*, 2017; Feng, 2022). Lignin, a structurally complex biopolymer, and tannins, a class of polyphenolic compounds, are degraded by certain microorganisms into smaller aromatic intermediates such as ferulic acid, gallic acid, vanillic acid, and salicylate (Janusz *et al.*, 2017; Sharma, 2019). Lignin and tannins comprise an estimated 30-40% of the global biomass, representing one of the largest reservoirs of aromatic carbon in the biosphere (Austin & Ballare, 2010; Arbenz & Avérous, 2015). Upon microbial depolymerization, these polymers release diverse phenolic compounds that must be efficiently catabolized to maximize carbon recovery and prevent toxic accumulation (Chauhan, 2020; Weng *et al.*, 2021).

In aerobic fungi and bacteria, the structurally diverse intermediates from lignin and other aromatic polymers degradation serve as a source of carbon and energy. Before ring cleavage and conversion to TCA cycle intermediates, these dihydroxylated aromatic compounds are funnelled into one of seven known central intermediates. In microorganisms, seven central aromatic ring-cleavage pathways have been described: the gentisate, gallic acid, hydroquinone, catechol, protocatechuate, hydroxyquinol and pyrogallol pathways (Harwood & Parales, 1996; Fuchs *et al.*, 2011; Lubbers *et al.*, 2019). Among these, the catechol, protocatechuate and hydroxyquinol pathways converge at the β -ketoadipate pathway, a major catabolic route through which different aromatic compounds, including benzoates and vanillate derivatives, are channelled into succinyl-CoA and acetyl-CoA, which subsequently feed into the TCA cycle in bacteria and fungi (Harwood & Parales, 1996; Diaz *et al.*, 2013; Lubbers *et al.*, 2019; Sgro *et al.*, 2023).

While the gentisate pathway and indeed all other pathways represent parallel ring-cleavage routes that catabolize hydroxylated aromatic intermediates, including hydroxybenzoates and hydroxyphenols (Romero-Silva *et al.*, 2013), they do not merge into the β -ketoadipate pathway (Fuchs *et al.*, 2011). Instead, they typically yield fumarate and pyruvate as central intermediates (Xu *et al.*, 2012). Together, these pathways form a network of converging catabolic routes that transform different aromatic compounds into common

metabolic intermediates, sustaining microbial growth and contributing to soil carbon turnover and nutrient regeneration (Harwood & Parales, 1996).

The sustainable valorization of lignocellulosic biomass represents one of the most pressing challenges in biotechnology, as it offers the potential to transform underutilized agricultural and forestry residues into valuable biochemicals while reducing environmental pollution (Singhvi & Gokhale, 2019). From a bioremediation perspective, the accumulation of aromatic compounds, particularly phenolic derivatives and polycyclic aromatic hydrocarbons, in contaminated environments poses significant ecological risks due to their persistence, toxicity, and antimicrobial properties. These compounds can disrupt soil and aquatic microbial communities, leading to reduced biodiversity and altered ecosystem functions, as many aromatics inhibit microbial growth and activity (Yang *et al.*, 2020; Hodson *et al.*, 2020).

Gentisate serves as an intermediate in the degradation of aromatic precursors such as salicylic acid, *p*-hydroxybenzoic acid, and 3-hydroxybenzoic acid (Grund *et al.*, 1990; Fairley *et al.*, 2006; Adams *et al.*, 2006). In bacteria, gentisate 1,2-dioxygenase catalyzes the ring cleavage oxidation of gentisate to form maleylpyruvate (Goetz & Harmuth, 1992; Xu *et al.*, 2012; Romero-Silva *et al.*, 2013). Maleylpyruvate can be directly hydrolyzed by maleylpyruvate hydrolase to maleate and pyruvate (Hopper *et al.*, 1971; Bayly *et al.*, 1980; Liu *et al.*, 2013) or isomerized to fumarylpyruvate, which is subsequently converted by fumarylpyruvate hydrolase to pyruvate and fumarate (Goetz & Harmuth, 1992; Zhou *et al.*, 2001).

Figure 2.1 shows the steps in the conversion of gentisate to pyruvate, maleate and fumarate in bacteria. The isomerization of maleylpyruvate to fumarylpyruvate is catalyzed by maleylpyruvate isomerase, which relies on low-molecular-weight thiol cofactors that play a role in facilitating the *cis-trans* rearrangement of the dicarboxylic acid. The content of low-molecular-weight thiols varies among phylogenetic groups of bacteria, with glutathione being dominant in Gram-negative bacteria, mycothiol the primary thiol in high G+C Gram-positive actinomycetes, and L-cysteine and coenzyme A the major thiols in low G+C Gram-positive bacteria (Fahey *et al.*, 1978; Newton *et al.*, 1996). Correlated to their thiol contents in bacteria is the identification of three phylogenetically unrelated maleylpyruvate isomerases: glutathione-dependent

maleylpyruvate isomerases in Gram-negative bacteria (Jones & Cooper, 1990; Goetz & Harmuth, 1992; Zhou *et al.*, 2001), mycothiol-dependent maleylpyruvate isomerases in actinomycetes (Hagedorn *et al.*, 1985; Shen *et al.*, 2005; Feng *et al.*, 2006), and a L-cysteine-dependent maleylpyruvate isomerase in the low-GC gram-positive bacterium *Paenibacillus* sp. (Liu & Zhou, 2012). These findings highlight the ability of bacteria to evolve multiple maleylpyruvate isomerases to make use of available thiols. Whereas the maleylpyruvate isomerases are phylogenetically unrelated among the different bacterial lineages, the other two gentisate catabolic enzymes, gentisate 1,2-dioxygenase and fumarylpyruvate hydrolase, are conserved. In addition, the genes encoding the gentisate catabolic enzymes in bacteria are organized in clusters despite the phylogenetic diversity of the maleylpyruvate isomerases (Liu & Zhou, 2012).

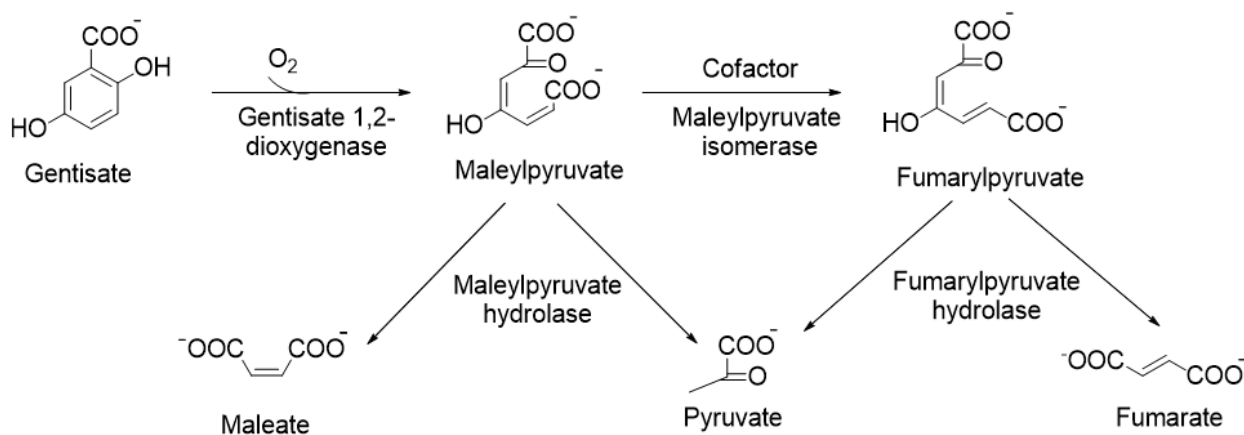


Figure 2.1 Catabolic pathway for the gentisate conversion to pyruvate, maleate, and fumarate in bacteria. Maleylpyruvate isomerase activity is dependent on either glutathione (GSH), mycothiol (MSH), L-cysteine, or cysteinylglycine as cofactor.

In the yeasts *Candida parapsilosis* and *Arxula adeninivorans*, glutathione accelerates the *in vitro* conversion of gentisate to pyruvate, leading to the suggestion that a glutathione-dependent maleylpyruvate isomerase is involved in the utilization of gentisate (Middelhoven *et al.*, 1992). A gene cluster in *C. parapsilosis* has been reported to be involved in gentisate catabolism based on comparative transcriptomic and proteomic analyses, and the presence of orthologues of the bacterial gentisate 1,2-dioxygenase and maleylpyruvate/fumarylpyruvate hydrolase (Holesova *et*

al., 2011; Cillingová *et al.*, 2022). Owing to the absence of a maleylpyruvate isomerase orthologue in the *C. parapsilosis* gentisate gene cluster and the sequence similarity between maleylpyruvate hydrolase and fumarylpyruvate hydrolase, it remains unclear whether gentisate is directly converted to maleate and pyruvate or to fumarate and pyruvate via isomerization of maleylpyruvate.

The filamentous fungus *Aspergillus niger* is a widely used cell factory for the production of proteins and organic acids (Cairns *et al.*, 2018; Cairns *et al.*, 2021). Its robust secretion systems, genetic tractability, and safety profile make it a cornerstone of modern biotechnology (Cairns *et al.*, 2018). With its well-annotated genome, this fungus has been used for the detailed analysis of metabolic pathways (Brandl *et al.*, 2018; Aguilar-Pontes *et al.*, 2018; Li *et al.*, 2022). As well, *A. niger* is known for its ability to break down aromatic compounds (Baker, 2006; Mäkelä *et al.*, 2015; Lubbers & de Vries, 2021), with the catabolic pathways characterized for phenol, *p*-hydroxybenzoic acid, protocatechuate, and benzoate (Supriya & Neehar, 2014; Semana & Powlowski, 2019; Lubbers, 2021). An expanded characterization of the catabolism of aromatics in *A. niger* is expected to fill our knowledge gap in the degradation of aromatics in fungi, as well as help to develop a platform for metabolic engineering applications in the valorization of lignocellulose.

In this study, we investigated the catabolism of gentisate in *A. niger*. Using orthology, transcriptome profiling, growth phenotypes, metabolite accumulation of knockout mutants, and gene complementation, we characterized a gene cluster involved in gentisate catabolism. We provided evidence to show that in *A. niger*, the conversion of gentisate involves the isomerization of maleylpyruvate. However, the *A. niger* orthologues of the bacterial glutathione-dependent maleylpyruvate isomerase are not involved in the gentisate catabolic pathway. We showed that a gene located in the gentisate gene cluster, phylogenetically unrelated to the characterized maleylpyruvate isomerases, is required for the *in vivo* conversion of maleylpyruvate to fumarylpyruvate.

2.3 Results.

2.3.1 Candidate genes of the gentisate pathway based on orthology and transcriptome analysis

To identify candidate genes encoding enzymes involved in the degradation of gentisate in *Aspergillus niger* based on sequence identity, we performed BLASTP searches against the *A. niger* NRRL3 genome (https://mycocosm.jgi.doe.gov/Aspni_NRRL3). As queries, we used the amino acid sequences of functionally characterized enzymes of the gentisate catabolic pathways from bacteria (Fahey *et al.*, 1978; Goetz & Harmuth, 1992). Table 2.1 shows the percentage sequence identity of *A. niger* orthologues of bacterial enzymes (cutoffs of >30% identity and >70% query coverage). Using the 355-amino acid sequence of gentisate 1,2-dioxygenase from *Ralstonia* sp. as a query, we identified two candidate proteins in *A. niger*, NRRL3_05236 and NRRL3_10244. These two proteins exhibit similar sequence identity with the *Ralstonia* protein, with NRRL3_05236 sharing 31% identity over 94% of its sequence and NRRL3_10244 showing 31% identity over 97%. Three phylogenetically unrelated maleylpyruvate isomerases, requiring different cofactors, have been reported for the bacterial gentisate catabolic pathways (Middelhoven *et al.*, 1992; Goetz & Harmuth, 1992; Fairley *et al.*, 2006; Liu & Zhou, 2012). Table 2.2 shows the pairwise sequence comparison of these three enzymes and their potential orthologues in *A. niger*. No orthologue was retrieved when the mycothiol-dependent or the cysteine-dependent enzymes were used as query sequences. However, the glutathione-dependent maleylpyruvate isomerase of *Ralstonia* sp. uncovered two *A. niger* orthologues NRRL3_04710 and NRRL3_09971. When fumarylpyruvate hydrolase from *Ralstonia* sp. or maleylpyruvate hydrolase from *Pseudomonas alcaligenes* were used as query sequences in BLASTP searches, each of them identified the same six orthologues in *A. niger* (Table 2.1). As such, we are unable to distinguish the orthologues of maleylpyruvate hydrolase from those of fumarylpyruvate hydrolase.

Table 2.1 Sequence identity of *A. niger* proteins to orthologues of bacterial gentisate catabolic enzymes.

Query sequence	<i>A. niger</i> orthologues	Sequence identity (%)
Gentisate 1,2-dioxygenase	NRRL3_05236	31
	NRRL3_10244	31
Maleylpyruvate isomerase	NRRL3_04710	36
	NRRL3_09971	40
Fumarylpyruvate hydrolase	NRRL3_02099	34
	NRRL3_05237	34
	NRRL3_05319	32
	NRRL3_08324	33
	NRRL3_10248	31
	NRRL3_11463	35
Maleylpyruvate hydrolase	NRRL3_02099	30
	NRRL3_05237	38
	NRRL3_05319	31
	NRRL3_08324	32
	NRRL3_10248	35
	NRRL3_11463	36

The amino acid sequences for gentisate 1,2-dioxygenase (GenBank accession number AAD12619.1), maleylpyruvate isomerase (GenBank accession number AAD12621.1), and fumarylpyruvate hydrolase (GenBank accession number AAD12620.1) from Ralstonia sp were used to search for their orthologues in A. niger. The A. niger orthologues of maleylpyruvate

hydrolase were identified using the amino acid sequence from *Pseudomonas alcaligenes* (GenBank accession number ABD64512.1).

Table 2.2 Pairwise sequence identity (%) between maleylpyruvate isomerases and their orthologues in *A. niger*

	Glutathione	Mycothiol	L-cysteine	NRRL3_04710	NRRL3_09971
Glutathione	100	17	16	39	34
Mycothiol		100	12	16	18
L-cysteine			100	13	16
NRRL3_04710				100	42
NRRL3_09971					100

The three maleylpyruvate isomerases used for comparison are denoted by their associated thiol cofactors. The GenBank accession numbers of these protein sequences are glutathione-dependent maleylpyruvate isomerase, AAD12621.1; mycothiol-dependent maleylpyruvate isomerase, WP_003855155; and cysteine-dependent maleylpyruvate isomerase, ADZ54051

Based on sequence similarity, *A. niger* possesses two or more candidate genes for each step of the gentisate catabolic pathway. To reduce the number of candidate genes for further examination, we used comparative transcriptomics to identify those that are differentially upregulated under growth on gentisate. We performed RNA-seq analysis of *A. niger* grown on gentisate or fructose as the sole carbon source. Table 2.3 summarizes the transcriptome results of the candidate genes identified based on sequence similarity and their neighbouring genes.

Both orthologues of the bacterial gentisate 1,2-dioxygenase gene, NRRL3_05236 and NRRL3_10244, were highly expressed during growth on gentisate (>6000 transcripts per million (TPM) for NRRL3_05236 and 232 TPM for NRRL3_10244), and they were significantly upregulated as compared to growth on fructose (0.1 TPM). Two of the six orthologues of maleylpyruvate/fumarylpyruvate hydrolase, NRRL3_05237 and NRRL3_10248, displayed

significant upregulation on gentisate, 14,278 TPM and 538 TPM, respectively, as compared to the 0.1 TPM when grown on fructose (Table 2.3). On the other hand, the two orthologues of the bacterial glutathione-dependent maleylpyruvate isomerases, NRRL3_04710 and NRRL3_09971, were not upregulated during growth on gentisate (Table 2.3).

Table 2.3 RNA-sequencing data for predicted gentisate degradation pathway genes in *A. niger*.

Description	Gene ID	Fructose (TPM)	Gentisate (TPM)
Gentisate 1,2-dioxygenase	<i>NRRL3_05236</i>	0.0	6066.1
	<i>NRRL3_10244</i>	0.1	231.8
Maleylpyruvate isomerase	<i>NRRL3_04710</i>	48.0	7.2
	<i>NRRL3_09971</i>	0.0	1.6
Fumarylpyruvate/maleylpyruvate hydrolase	<i>NRRL3_02099</i>	35.2	93.9
	<i>NRRL3_05237</i>	0.1	14728.4
	<i>NRRL3_05319</i>	99.7	183.1
	<i>NRRL3_08324</i>	2.6	2.2
	<i>NRRL3_10248</i>	0.1	538.1
	<i>NRRL3_11463</i>	1.4	6.7
Fungal-specific transcription factor	<i>NRRL3_05233</i>	0.8	491.3
	<i>NRRL3_10246</i>	5.4	12.5
*Alcohol dehydrogenase	<i>NRRL3_05234</i>	0.2	3320.3
*Glutathione-dependent formaldehyde-activating family protein	<i>NRRL3_05235</i>	0.2	6630.3
*Aromatic-ring hydrolase	<i>NRRL3_10245</i>	0.3	145.4
* β -carotene-cleaving oxygenase	<i>NRRL3_10247</i>	7.1	159.7
*Major facilitator superfamily protein	<i>NRRL3_10249</i>	1.9	118.3
*Lysophospholipase catalytic domain-containing protein	<i>NRRL3_10250</i>	46.4	357.7

**These genes are co-localized with predicted gentisate pathway genes, but their function in the pathway is unknown. TPMs, transcripts per million, are the mean of duplicate determinations.*

Figure 2.2 shows that, with the exception of the orthologues of the maleylpyruvate isomerase, the candidate genes for the gentisate catabolic pathways are localized in two clusters of the genome. Based on transcription upregulation on gentisate, the *NRRL3_05233-NRRL3_05237* cluster on Chromosome_4 includes genes predicted to encode a fungal-specific transcription factor (*NRRL3_05233*), a zinc-type alcohol dehydrogenase (*NRRL3_05234*), a glutathione-dependent formaldehyde-activating family protein (*NRRL3_05235*), in addition to the candidate gentisate 1,2-dioxygenase (*NRRL3_05236*) and the maleylpyruvate/fumarylpyruvate hydrolase (*NRRL3_05237*). Likewise, the other cluster on Chromosome_8 that includes the candidate genes for gentisate 1,2-dioxygenase (*NRRL3_10244*) and maleylpyruvate/fumarylpyruvate hydrolase (*NRRL3_10248*) extends from *NRRL3_10244* to *NRRL3_10250* (Figure 2.2). The genes within these two clusters, with the exception of *NRRL3_10246* encoding a fungal-specific transcription factor, were significantly upregulated during growth on gentisate (Table 2.3). The genes encoding the orthologues of maleylpyruvate isomerase, *NRRL3_04710* and *NRRL3_09971*, are located off-cluster on Chromosome_4 and Chromosome_7, respectively.

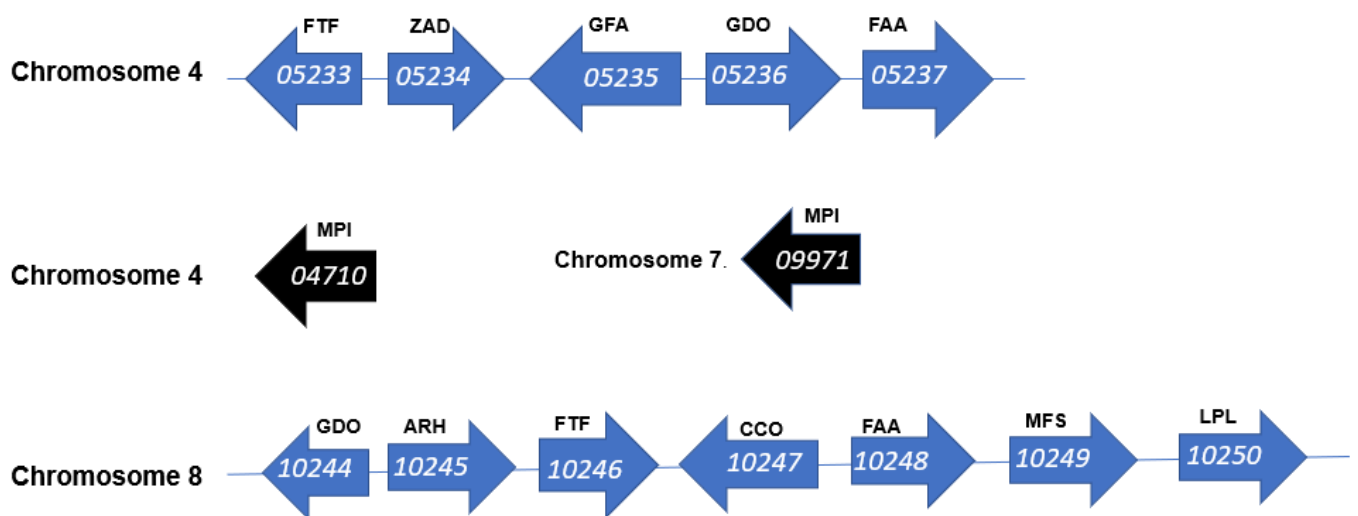


Figure 2.2. Organization of genes encoding predicted gentisate catabolic enzymes and co-localized, upregulated genes. Genes predicted to encode gentisate catabolic enzymes, with abbreviations based on protein domain analysis and orthology: *ARH*, aromatic-ring hydrolase;

CCO, carotenoid-cleaving oxygenase; *FAA*, fumarylacetoacetase family protein; *FTF*, fungal-specific transcription factor; *GDO*, gentisate 1,2-dioxygenase; *GFA*, glutathione-dependent formaldehyde-activating family protein; *LPL*, lysophospholipase catalytic domain-containing protein; *MFS*, major facilitator superfamily protein; *MPI*, maleylpyruvate isomerase; and *ZAD*, zinc-type alcohol dehydrogenase. Gene ID numbers correspond to *A. niger* NRRL3 annotations.

2.3.2 The orthologues of the bacterial maleylpyruvate isomerase are not essential for the catabolism of gentisate

One of the questions that we set out to address in this study is to determine whether the fungal degradation of gentisate to pyruvate follows through the hydrolysis of maleylpyruvate to maleate and pyruvate or the indirect pathway via the isomerization of maleylpyruvate to fumarylpyruvate with its subsequent hydrolysis to fumarate and pyruvate. We identified two orthologues of the bacterial maleylpyruvate isomerase in *A. niger*, NRRL3_04710 and NRRL3_09971 (Table 2.1). To determine the potential role of these two orthologues in gentisate catabolism, we constructed the following *A. niger* deletion strains: Δ NRRL3_04710, Δ NRRL3_09971, and the double deletion Δ NRRL3_04710/ Δ NRRL3_09971 (Figures S2.1-2.3). All three mutant strains showed growth on gentisate equal to that of the parent strain (Figure 2.3). These observations, along with them not being upregulated when grown on gentisate (Table 2.3), suggest that NRRL3_04710 and NRRL3_09971 are not essential for the utilization of gentisate in *A. niger*. These results imply that if the utilization of gentisate in *A. niger* follows the indirect pathway, a novel enzyme or mechanism is involved in the isomerization of maleylpyruvate to fumarylpyruvate.

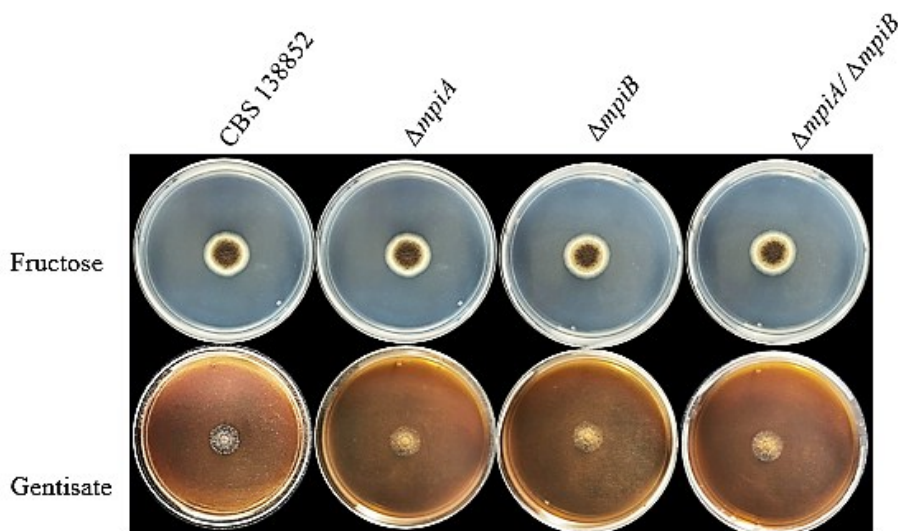


Figure 2.3 Growth phenotype of *A. niger* parent strain (CBS 138852) and predicted maleylpyruvate isomerase deletion mutants: ΔmpiA ($\Delta\text{NRRL3_04710}$), ΔmpiB ($\Delta\text{NRRL3_09971}$), and the double deletion $\Delta\text{mpiA}/\text{mpiB}$ ($\Delta\text{NRRL3_04710}/\Delta\text{NRRL3_09971}$) mutants. Strains were cultivated on minimal medium (MM) supplemented with fructose or gentisate as carbon sources.

2.3.3 Cluster of genes involved in gentisate catabolism

As shown in Figure 2.2, two gene clusters of *A. niger* harbour orthologues of bacterial gentisate catabolic genes. To determine whether the orthologues in either one or both of these gene clusters are involved in gentisate catabolism, we constructed strains with the predicted enzyme-encoding genes of these two clusters deleted: $\Delta\text{NRRL3_05234-NRRL3_05237}$ and $\Delta\text{NRRL3_10244-NRRL3_10250}$ (Figures S2.4-2.5). Figure 2.4 shows that $\Delta\text{NRRL3_05234-NRRL3_05237}$ was unable to grow on gentisate medium, while its growth on fructose remained unaffected. However, the $\Delta\text{NRRL3_10244-NRRL3_10250}$ mutant grew as well as the parent strain on either gentisate or fructose. These results suggest that the $\text{NRRL3_05234-NRRL3_05237}$ cluster harbours genes involved in gentisate catabolism, whereas the orthologues in the $\text{NRRL3_10244-NRRL3_10250}$ cluster do not play a role in gentisate catabolism.

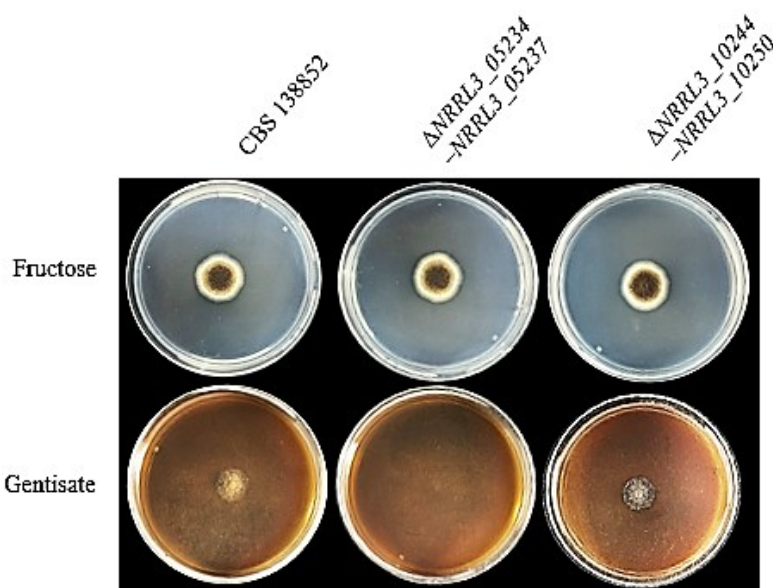


Figure 2.4 Growth phenotypes of *A. niger* parent strain (CBS 138852) and predicted gentisate cluster deletion mutants. Strains were cultivated on fructose or gentisate.

To determine the physiological role of the genes in the NRRL3_05233–NRRL3_05237 cluster, we constructed strains with deletions in each of the genes (Figures S2.6-2.10). Based on protein domain analysis, NRRL3_05233 is predicted to encode a Zn2Cys6 class of fungal transcription factor. Strain Δ NRRL3_05233 displayed reduced growth on gentisate when compared to the parent (Figure 2.5). This result, together with the protein domain analysis, suggests that NRRL3_05233 plays a role in the regulation of gentisate catabolic genes. We therefore name NRRL3_05233 as *gtaR*. However, one or more other regulators are likely to be involved in gentisate catabolism because Δ *gtaR* showed partial growth on gentisate.

Protein domain analysis suggests that NRRL3_05234 encodes a zinc-binding alcohol dehydrogenase. If the fungal gentisate catabolic pathway resembles that of bacterial pathways, alcohol dehydrogenase is not expected to be involved. As expected, no altered phenotype was observed when Δ NRRL3_05234 was cultured on gentisate (Figure 2.5).

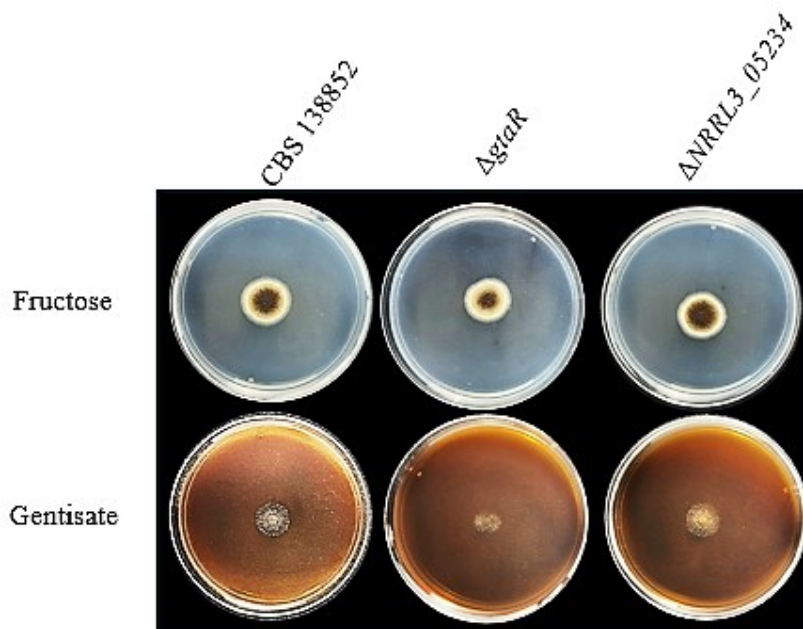


Figure 2.5 Growth phenotypes of *A. niger* parent strain (CBS 138852), with co-localized pathway genes, $\Delta gtaR$ ($\Delta NRRL3_05233$) and $\Delta NRRL3_05234$. Strains were cultivated on fructose or gentisate.

As an orthologue of the bacterial gentisate 1,2-dioxygenase, NRRL3_05236 is predicted to catalyze the first step on the gentisate pathway to yield the ring-cleavage product maleylpyruvate. Deletion of $\Delta NRRL3_05236$ resulted in complete loss of growth on gentisate medium (Figure 2.6A), indicating that NRRL3_05236 is essential for the catabolism of gentisate in *A. niger*. Metabolite analysis showed that the parent strain (CBS 138852) consumed most of the gentisate within 24 hours. The consumption of gentisate in the $\Delta NRRL3_05236$ was minimal over 48 hours of cultivation, as expected for a mutant lacking gentisate 1,2-dioxygenase (Figure 2.6B). Mutant characteristics and sequence similarity support the conclusion that NRRL3_05236 encodes the ring-cleavage gentisate 1,2-dioxygenase, and we name it *gdoA*.

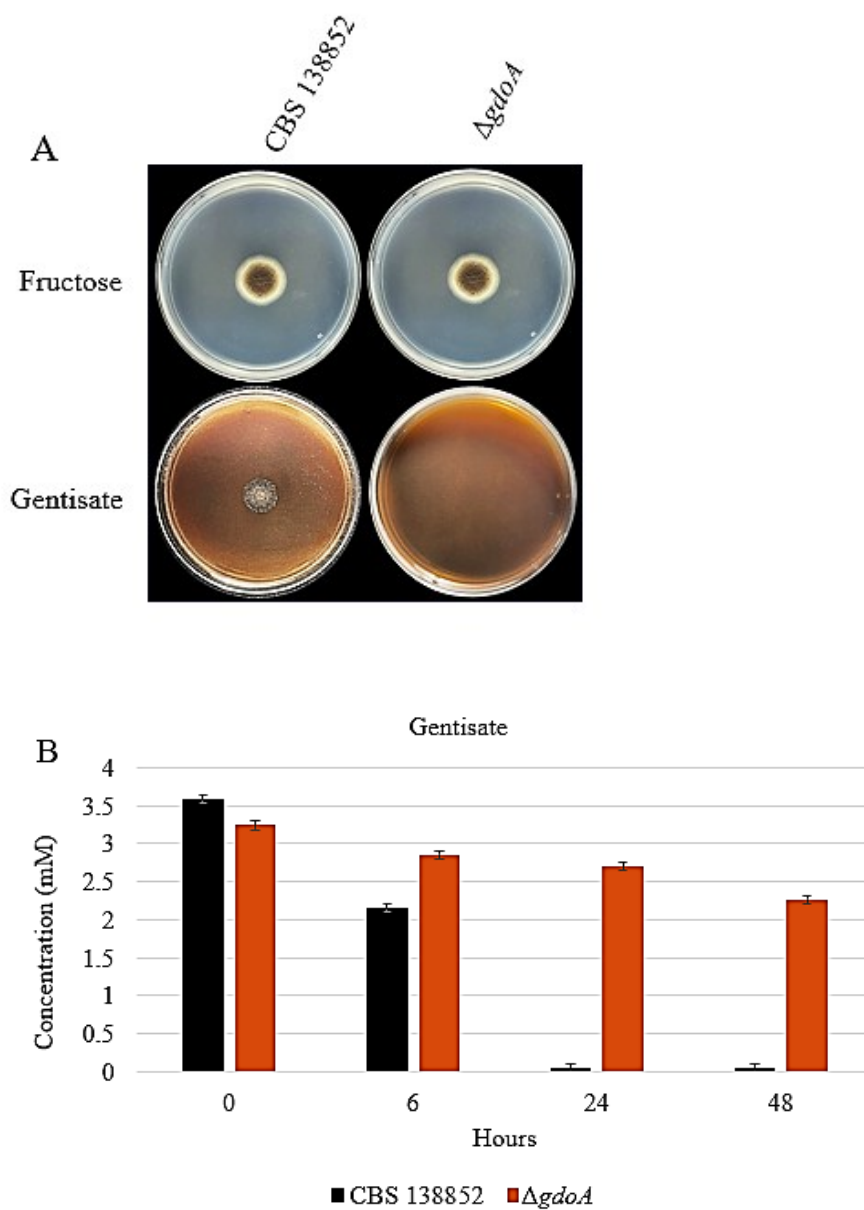
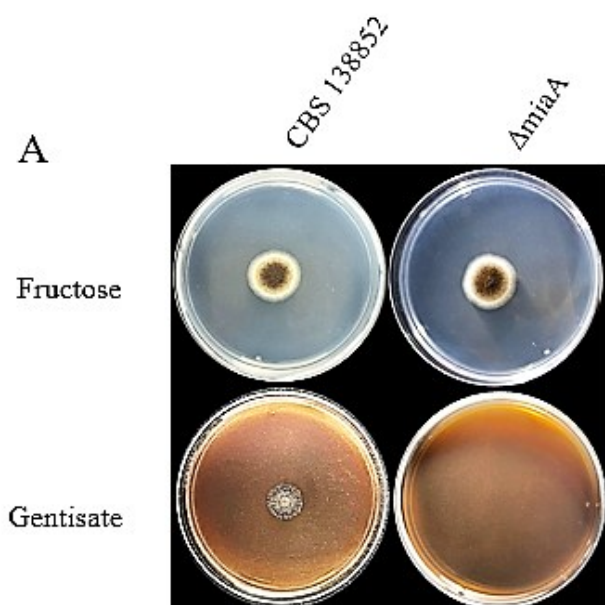


Figure 2.6 Growth phenotype and gentisate utilization of *Aspergillus niger* parent strain CBS 138852 and the gentisate 1,2-dioxygenase deletion mutant $\Delta gdoA$ ($\Delta NRRL3_05236$). (A) Growth of the parent strain and the $\Delta gdoA$ mutant on fructose or gentisate as the sole carbon source. (B) Time-course of gentisate consumption during cultivation on gentisate. Gentisate concentrations were quantified by LC-MS in negative ionization mode. Data represent the mean of duplicate cultures for each strain.

NRRL3_05235 contains a centromere protein V/glutathione-dependent formaldehyde activating enzyme (CENP-V/GFA) domain (Prosite PS51891), a conserved domain implicated in chromosome condensation in mammalian cells (Tadeu *et al.*, 2008) and glutathione-dependent detoxification of formaldehyde in prokaryotes (Hopkinson *et al.*, 2015). Figure 2.7A shows that Δ NRRL3_05235 exhibited growth failure on gentisate medium. The growth defect of Δ NRRL3_05235 indicates that NRRL3_05235 is required for the utilization of gentisate in *A. niger*. In the analysis of pathway mutants, when the gene encoding a pathway enzyme is knocked out, we can expect to detect the accumulation of the product of its preceding enzymatic step (Alazi *et al.*, 2016). We used liquid chromatography-mass spectrometry (LC-MS) to analyze metabolites in extracellular media accumulated after cultivating Δ NRRL3_05235 mutants on gentisate. While the rate of gentisate utilization in Δ NRRL3_05235 was slower than that of the parent strain, with a product accumulation (Figures 2.7B-C), the MS profile (Figure 2.8) shows a peak with $m/z = 185.0091$ ([M-H]⁻)—that corresponds to maleylpyruvate in the mutant strain but is absent in the parent strain. The detected compound eluted at a retention time of 3.9 minutes, reaching its highest level at 24 hours of cultivation (Figure 2.7C).



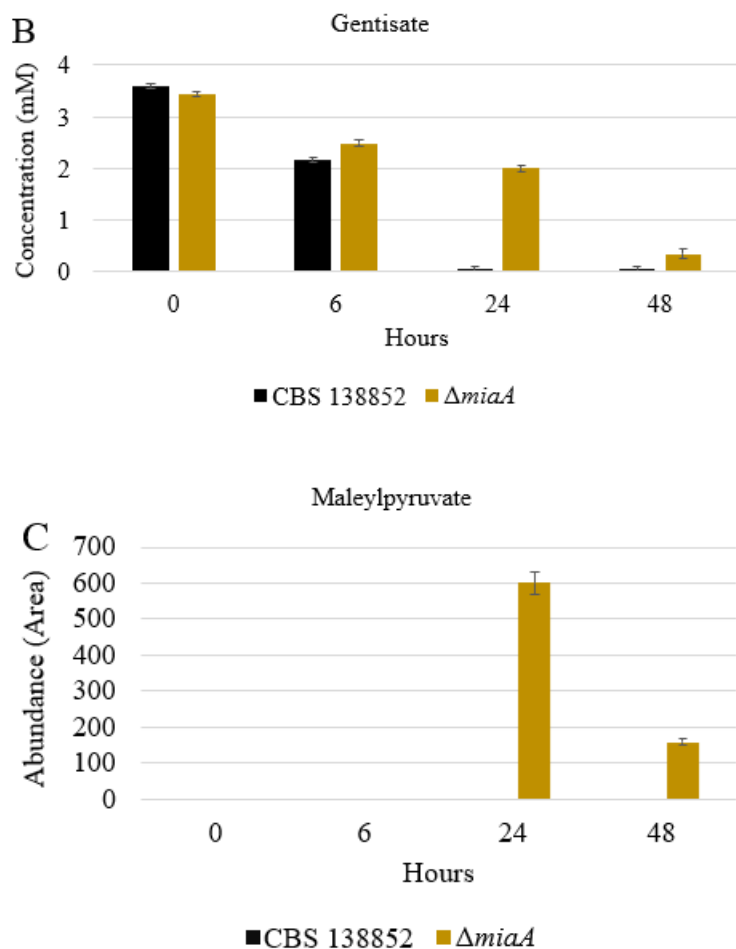


Figure 2.7 Growth phenotype and metabolic profiling of *Aspergillus niger* CBS 138852 and the deletion mutant $\Delta miaA$ (NRRL3_05235). (A) Growth of the parent strain CBS 138852 and the $\Delta miaA$ mutant on minimal medium containing fructose or gentisate as the sole carbon source. (B) Time-course of gentisate consumption during cultivation on gentisate by the parent and $\Delta miaA$ strains. (C) Accumulation of the maleylpyruvate intermediate in the parent and $\Delta miaA$ strains by LC–MS in negative ionization mode. Data represent mean values from replicate cultures.

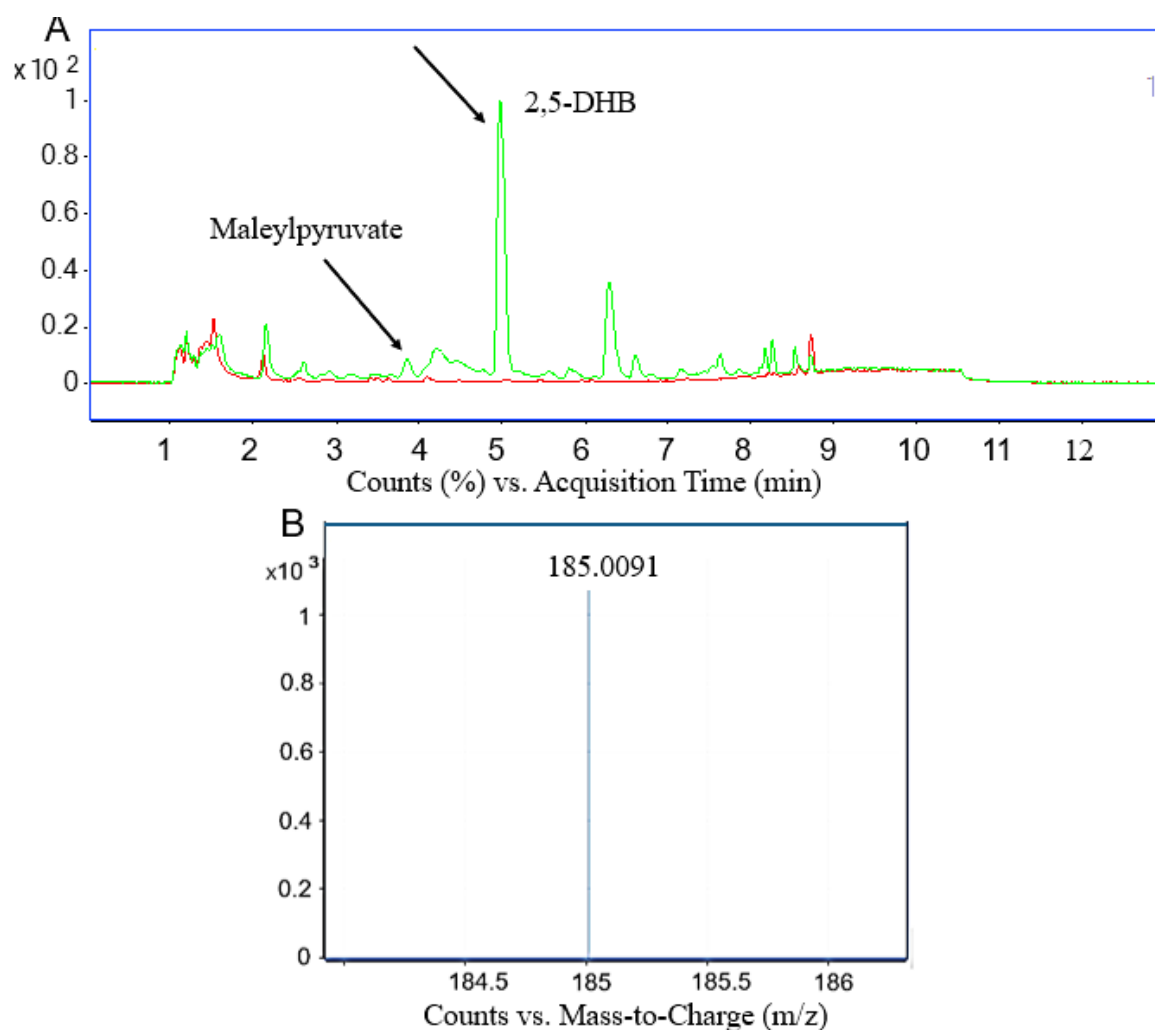


Figure 2.8 LC-MS analysis of culture supernatants from $\Delta miaA$ ($\Delta NRRL3_05235$) and *A. niger* parent strain (CBS 138852) grown on gentisate. (A) Total ion chromatograms of culture supernatants from $\Delta miaA$ (green trace) and parent strain (red trace) after 48 hours of growth, showing a peak corresponding to maleylpyruvate. (B) Mass spectrum of the peak at ~3.9 minutes retention time in the $\Delta miaA$ strain.

To further verify the identity of the accumulated compound, the LC-MS/MS fragmentation pattern of *in-vitro*-generated maleylpyruvate was analyzed and used as a reference compound for comparison to the fragmentation of the metabolite accumulated in $\Delta miaA$. The mass spectra from both conditions displayed identical fragmentation patterns, with matching m/z values at 69.034,

97.029, 113.025, and approximately 141. This indicates that the accumulated metabolite in the mutant strain is maleylpyruvate (Figure 2.9).

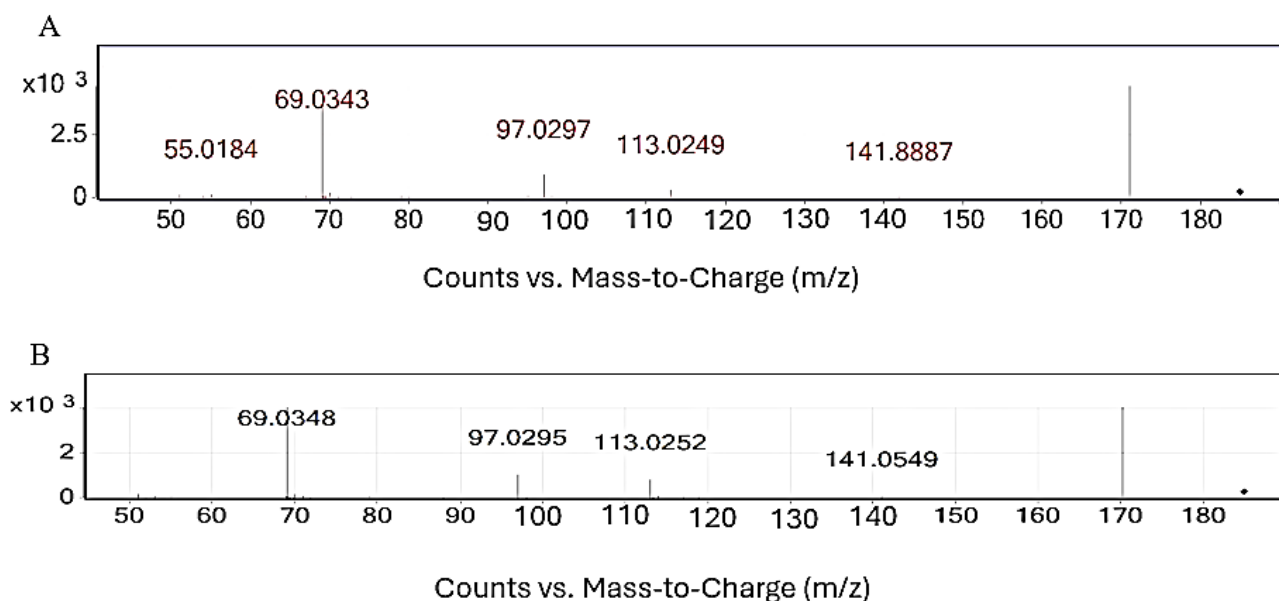


Figure 2.9 LC-MS/MS fragmentation of the pathway intermediate, compared to the reference compound. (A) Fragmentation pattern of in vitro-generated maleylpyruvate (B) fragmentation pattern of the metabolite accumulated in the NRRL3_05235 ($\Delta miaA$) strain.

2.3.4 Loss-of-function of NRRL3_05235 can be functionally complemented by maleylpyruvate isomerase genes

To further investigate the role of NRRL3_05235 in gentisate catabolism and its relationship with maleylpyruvate isomerase, we replaced NRRL3_05235 with the bacterial glutathione-dependent maleylpyruvate isomerase gene (*nagL*) from *Ralstonia* sp., as well as with its orthologues in *A. niger*, NRRL3_04710 and NRRL3_09971. All three mutants, $\Delta NRRL3_05235::nagL$, $\Delta NRRL3_05235::NRRL3_04710$ and $\Delta NRRL3_05235::NRRL3_09971$, displayed growth on gentisate or fructose comparable to that of the parent strain (Figure 2.10). Conclusions that can be drawn from the gene replacement experiments are twofold. First, the function of NRRL3_05235 can be replaced by maleylpyruvate isomerase. In other words, NRRL3_05235 plays a role in maleylpyruvate isomerization to fumarylpyruvate. However, unlike

its prokaryotic orthologue, which acts as a glutathione carrier rather than a direct biocatalyst, NRRL3_05235 likely activates the reaction in *A. niger* (Hopkinson *et al.*, 2015). We refer to NRRL3_05235 as a maleylpyruvate isomerization-activating protein with its encoding gene as *miaA*. Second, the orthologues NRRL3_04710 and NRRL3_09971 are structurally and functionally similar to the *Ralstonia* maleylpyruvate isomerase (*nagL*) gene. We therefore refer to NRRL3_04710 and NRRL3_09971 as *mpiA* and *mpiB*, respectively.

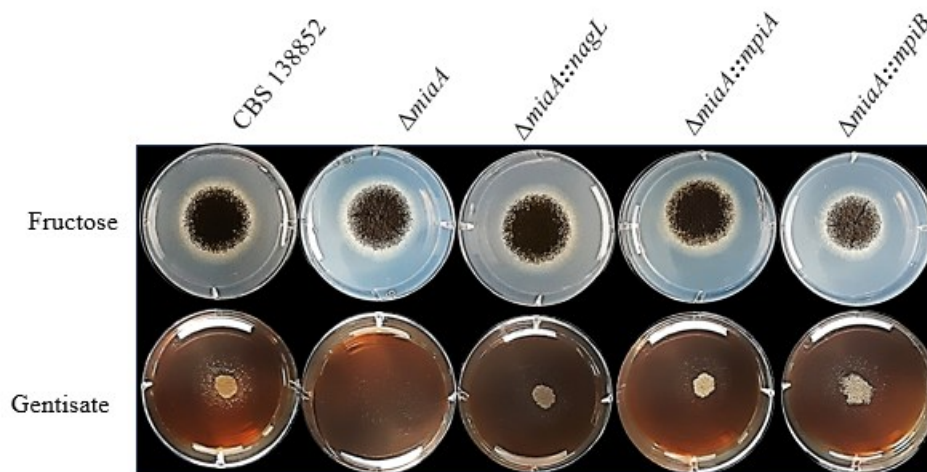


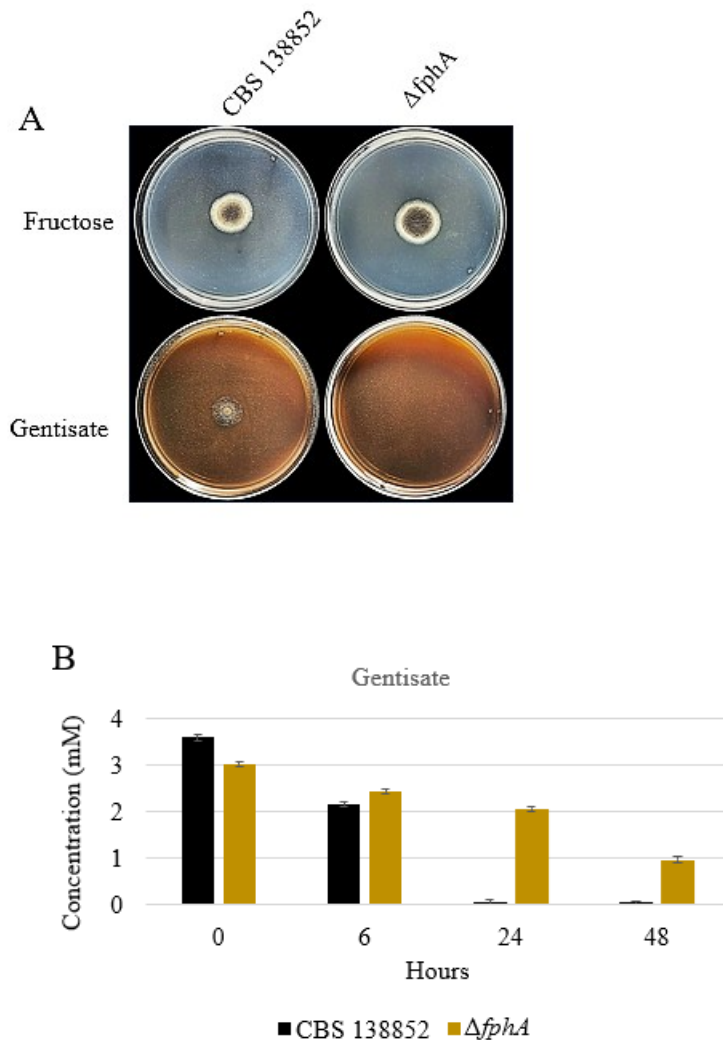
Figure 2.10 Growth phenotype of functionally complemented $\Delta miaA$ ($\Delta NRRL3_05235$) strains in *A. niger*. Growth of $\Delta miaA$ strains complemented with bacterial maleylpyruvate isomerase *nagL* or *A. niger* orthologues *mpiA* (NRRL3_04710) and *mpiB* (NRRL3_09971).

2.3.5 NRRL3_05237 is required for the catabolism of gentisate

Orthology analysis suggests that NRRL3_05237 is a maleylpyruvate/fumarylpyruvate hydrolase. Deletion of NRRL3_05237 completely abolished growth on gentisate, indicating that this gene is required for the utilization of gentisate (Figure 2.11A). Liquid chromatography–mass spectrometry analysis of culture supernatants collected at different time points showed that the $\Delta NRRL3_05237$ mutant consumed gentisate at a markedly slower rate than the parent strain, consistent with disruption of a gene encoding a pathway enzyme. In addition, a metabolite was detected exclusively in the mutant strain, $\Delta NRRL3_05237$ (*fphA*) (Figure 2.11B–C). The MS profile (Figure 2.12) revealed a distinct peak at m/z 185.0092 corresponding to fumarylpyruvate

that accumulated in the $\Delta NRRL3_05237$ mutant at 24 hours of cultivation. Although this metabolite was no longer detected at 48 hours in the mutant, it was completely absent from the parent strain (Figure 2.11C), further supporting impaired gentisate catabolism in $\Delta NRRL3_05237$.

With the small quantity accumulated in $\Delta NRRL3_05237$, we were unable to obtain an MS/MS fragmentation pattern of the m/z 185.0092 compound. Although the compound detected in $\Delta NRRL3_05237$ had the same mass as that observed in $\Delta NRRL3_05235$, the accumulated metabolite in $\Delta NRRL3_05235$ eluted at a retention time of 3.9 min. In contrast, the corresponding metabolite in $\Delta NRRL3_05237$ eluted at 2.3 min. (Figure 2.12).



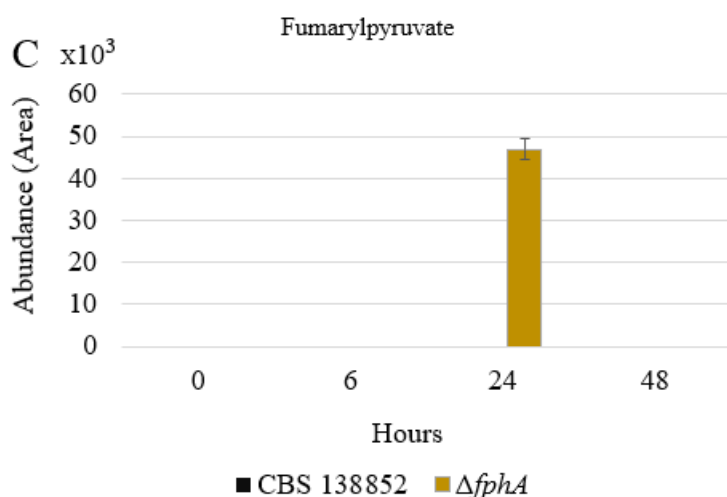


Figure 2.11 Growth phenotype and gentisate catabolism in *Aspergillus niger* parent strain (CBS 138852) and fumarylpyruvate hydrolase deletion mutant $\Delta fphA$ ($\Delta NRRL3_05237$). (A) Growth of the parent strain and $\Delta fphA$ mutant on fructose or gentisate as the sole carbon source. (B) Quantification of gentisate consumption over time in culture supernatants of the parent strain and $\Delta fphA$ mutant as determined by LC–MS. (C) LC–MS analysis (negative ion mode) showing accumulation of fumarylpyruvate metabolite in the $\Delta fphA$ mutant, which was absent in the parent strain. Data represent mean values from replicate cultures.

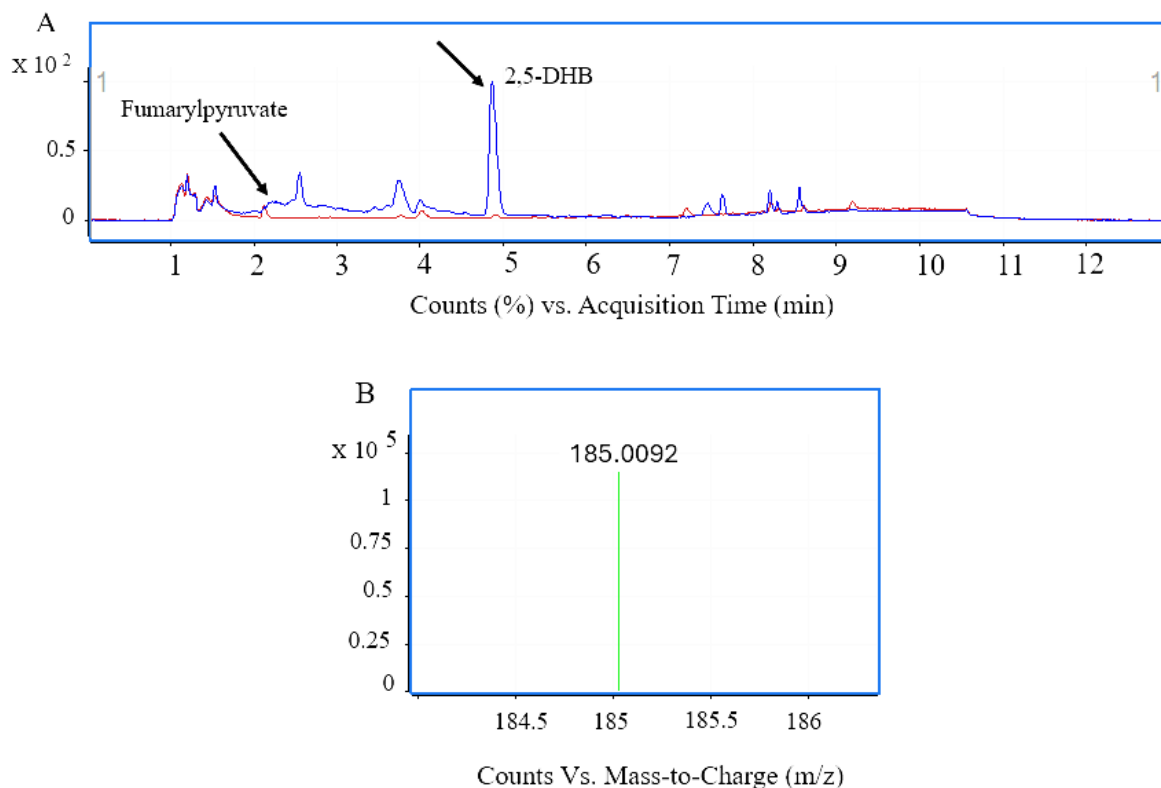


Figure 2.12 LC-MS Analysis of culture supernatants from $\Delta fphA$ ($\Delta NRRL3_05237$) and *A. niger* parent strain (CBS 138852) grown on gentisate. (A) Total ion chromatograms of culture supernatants from $\Delta fphA$ (blue trace) and parent (red trace) strains at 24 hours of incubation, showing a peak corresponding to fumarylpyruvate. (B) Mass spectrum of the fumarylpyruvate peak at 2.3 minutes retention time in the $\Delta fphA$ strain.

To complement our earlier findings and gain a more comprehensive understanding of the product accumulated in the $\Delta NRRL3_05237$ strain, we used an alternative approach. Using the same replacement gene cassettes described in this study, we replaced the disrupted $\Delta miaA$ locus in $\Delta NRRL3_05237$ strains with either the *nagL* gene (encoding a maleylpyruvate isomerase from *Ralstonia* sp.) or the *A. niger* orthologues, *mpiA* or *mpiB*. Growth was completely abolished when these mutant strains were cultivated on solid gentisate medium, as expected for mutants lacking FphA activity (Figure 2.13). Since maleylpyruvate isomerase is involved in gentisate catabolism, fumarylpyruvate is a predicted intermediate of the pathway. Therefore, we hypothesized that loss of FphA activity would lead to the accumulation of fumarylpyruvate. LC-MS analysis of culture supernatants revealed that these double mutant strains ($\Delta miaA::nagL \Delta fphA$, $\Delta miaA::mpiA \Delta fphA$,

$\Delta miaA::mpiB \Delta fphA$), engineered to express functionally characterized bacterial MPI or its predicted *A. niger* orthologues at the disrupted *miaA* locus, revealed the accumulation of an unknown compound that was not detected in the parent strain (Figure 2.14 B-D).

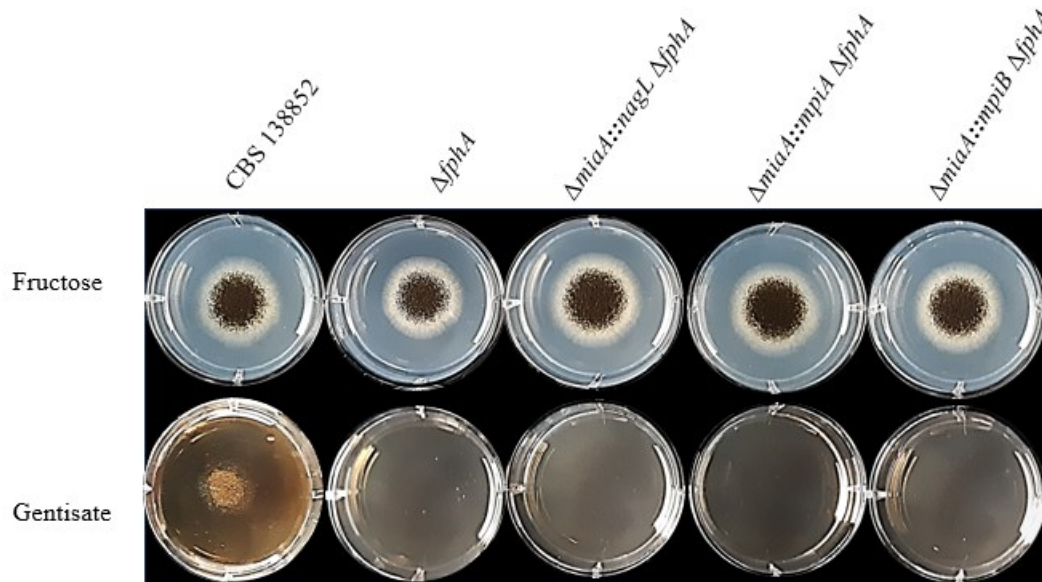
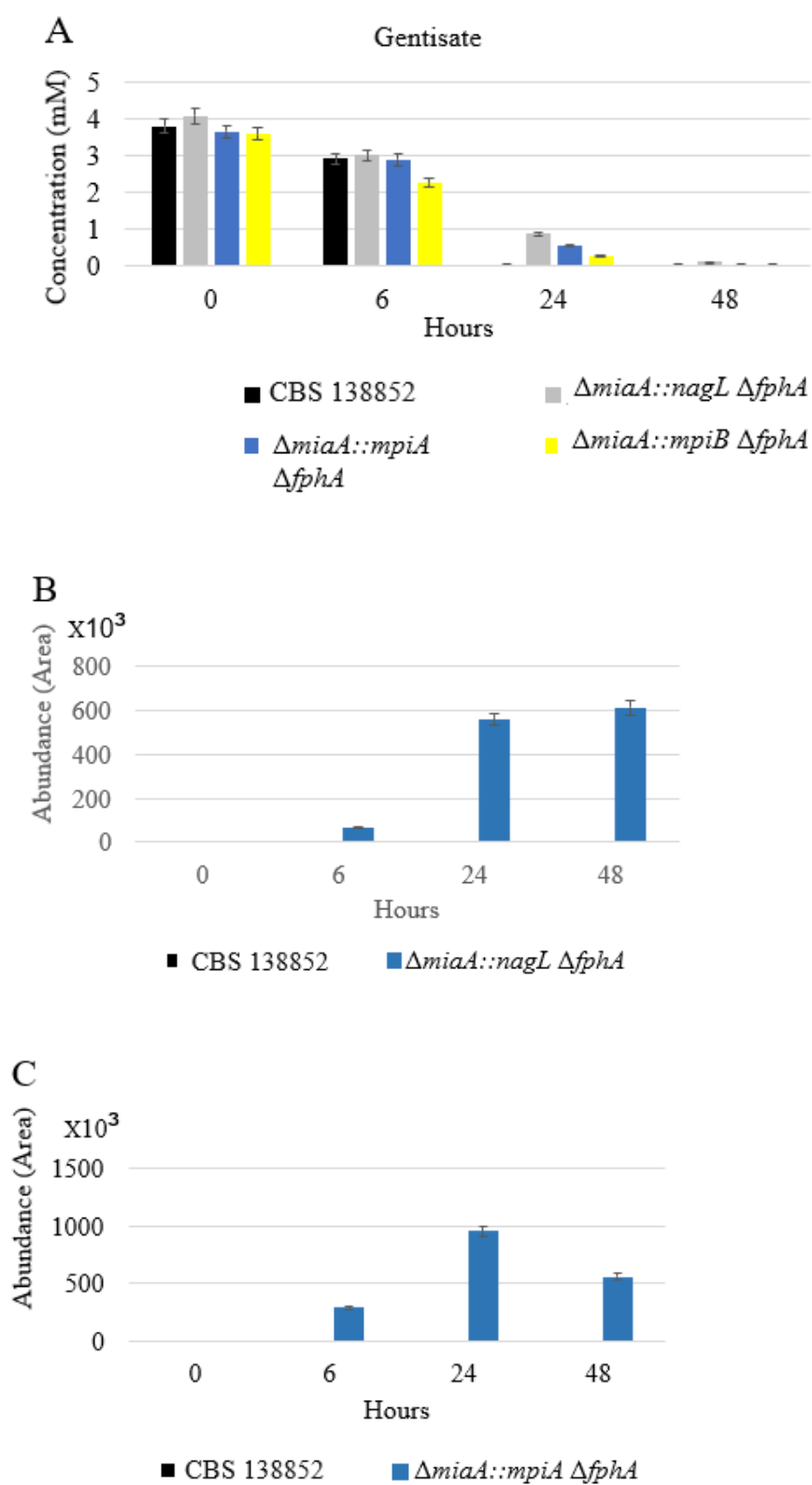


Figure 2.13 Growth phenotype of disrupted $\Delta miaA$ replaced with all MPIs in $\Delta fphA$ ($\Delta NRRL3_05237$) strains. Growth phenotype of $\Delta fphA$ strains with bacterial maleylpyruvate isomerase, *nagL* or *A. niger* orthologues *mpiA* and *mpiB* in the $\Delta miaA$ locus in the $\Delta fphA$ strain on agar plates.



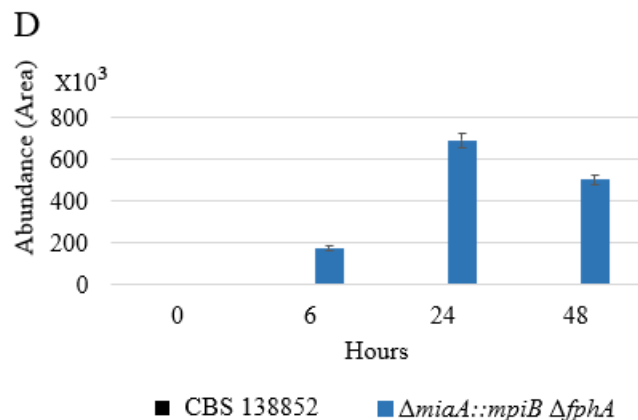


Figure 2.14 LC-MS analysis of gentisate utilization in $\Delta fphA$ ($\Delta NRRL3_05237$) strains with $\Delta miaA$ replaced with MPIs. (A) Utilization of gentisate by *A. niger* parent strain (CBS 138852) and $\Delta fphA$ strains carrying replacement genes in the $\Delta miaA$ locus. (B–D) Accumulation of unidentified metabolites in $\Delta miaA::nagL \Delta fphA$ (B), $\Delta miaA::mpiA \Delta fphA$ (C), and $\Delta miaA::mpiB \Delta fphA$ (D).

As no standard was available for fumarylpyruvate, its identity was inferred using the Agilent MassHunter Molecular Structure Correlator Software. For this analysis, only the $\Delta NRRL3_05237$ strain engineered to express a functionally characterized bacterial maleylpyruvate isomerase (Figure 2.14 B) was examined. The software predicted fumarylpyruvate eluting at 2.3 minutes as one of the most likely candidates based on the observed MS/MS fragmentation spectrum (Figure 2.15). With its requirement in gentisate catabolism (Figure 2.11 A) and sequence similarity to the bacterial maleylpyruvate and fumarylpyruvate hydrolases (Table 2.1), $NRRL3_05237$ most likely encodes fumarylpyruvate hydrolase. We therefore name $NRRL3_05237$ as *fphA*.

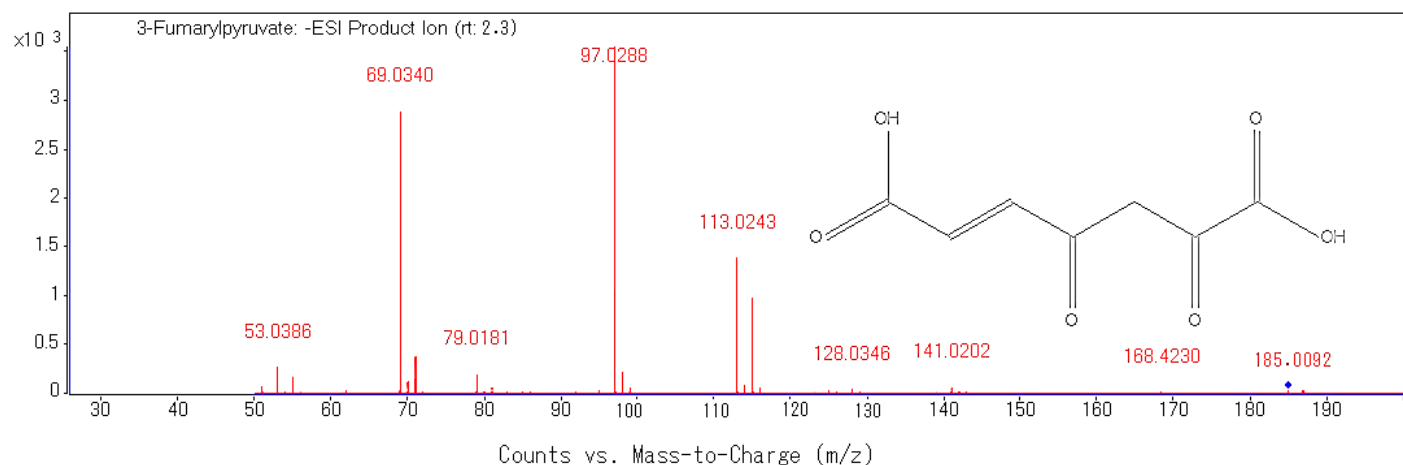


Figure 2.15 Molecular structure correlator identification of fumarylpyruvate as the best match to the MS/MS fragmentation profile of the ion at m/z 185.0092 (retention time 2.3 min) in the $\Delta miaA::nagL \Delta phA$ strain.

2.4 Discussion

Gentisate is a catabolic intermediate of aromatic compounds. In the bacterium *Ralstonia* *sp.* strain U2, gentisate is converted to the TCA intermediates pyruvate and fumarate by the following steps: gentisate to maleylpyruvate by the enzyme gentisate 1,2-dioxygenase, maleylpyruvate to fumarylpyruvate by maleylpyruvate isomerase, and fumarylpyruvate to pyruvate and fumarate by fumarylpyruvate hydrolase (Zhou *et al.*, 2001). In the bacterium *Pseudomonas alcaligenes*, however, the isomerization step can be omitted because maleylpyruvate can be directly hydrolyzed to pyruvate and maleate by maleylpyruvate hydrolase (Liu *et al.*, 2013). The bacterial pathways are summarized in Figure 2.1.

Using cellular extracts of the yeasts *C. parapsilosis* and *A. adenivorans* to hydrolyze 3-hydroxybenzoate, the precursor of gentisate, the rate of conversion from gentisate to pyruvate was accelerated by glutathione (Middelhoven *et al.*, 1992). Since the glutathione was known at the time to be a cofactor of the enzyme converting maleylpyruvate to fumarylpyruvate (Lack, 1961), Middelhoven *et al.* (1992) proposed that the catabolism of gentisate in yeasts involves the isomerization of maleylpyruvate to fumarylpyruvate. In other words, gentisate catabolism in yeasts involves a three-step process: (1) gentisate 1,2-dioxygenase cleaves the gentisate ring to form maleylpyruvate, (2) maleylpyruvate isomerase converts maleylpyruvate to fumarylpyruvate, and

(3) fumarylpyruvate hydrolase hydrolyzes fumarylpyruvate. The genes involved in the catabolism of 3-hydroxybenzoate, including those predicted to encode gentisate catabolic enzymes, are co-localized in the *C. parapsilosis* genome (Holesova *et al.*, 2011). Based on transcriptome and proteomic profiles of *C. parapsilosis* cells grown on 3-hydroxybenzoate and protein domain analysis, the genes encoding gentisate catabolic enzymes, gentisate 1,2-dioxygenase, maleylpyruvate isomerase and fumarylpyruvate hydrolase, were assigned (Cillingová *et al.*, 2022). However, the proposed *C. parapsilosis* maleylpyruvate isomerase does not share protein domain or sequence similarity to known maleylpyruvate isomerases.

In this study, we functionally examined the role of a gene cluster in *A. niger* involved in the catabolism of gentisate. Proteins encoded by the *A. niger* gentisate gene cluster are orthologues of the *C. parapsilosis* 3-hydroxybenzoate gene cluster (Figure 2.16).

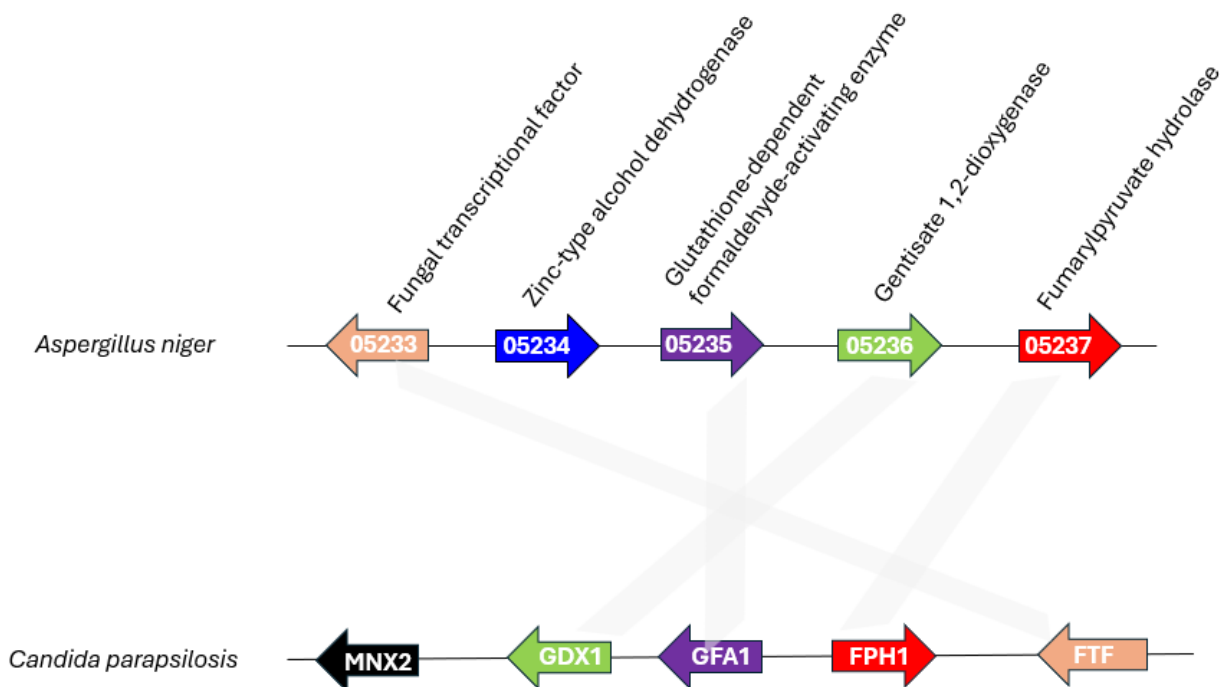


Figure 2.16 Comparative organization of gentisate catabolic pathway genes in *Aspergillus niger* and *Candida parapsilosis*. Schematic showing homologous genes within the predicted gentisate catabolic clusters of *A. niger* and *C. parapsilosis*, connected by shaded grey blocks. Numbers

within arrows indicate *A. niger* gene ID numbers (NRRL3_05233–NRRL3_05237). The corresponding orthologues in *C. parapsilosis* are labelled **FTF**, **GFA**, **FPH1**, and **GDX1**, with GenBank accession numbers **GDX1** (KAI5908751.1), **FPH1** (KAI5908750.1), **GFA1** (KAI5908749.1), and **FTF** (KAI5908748.1). Gene **MNX2** (3-hydroxybenzoate 6-hydroxylase) is co-localized within the *C. parapsilosis* cluster. Homology was determined by BLASTP, with amino acid sequence (%) identity/query coverage as follows: **FTF**–NRRL3_05233, 30/91; **GFA**–NRRL3_05235, 38/98; **GDX1**–NRRL3_05236, 53/98; and **FPH1**–NRRL3_05237, 58/98.

Unlike the *C. parapsilosis* cluster, the *A. niger* cluster does not contain the gene encoding 3-hydroxybenzoate 6-hydroxylase responsible for the conversion of 3-hydroxybenzoate to gentisate (Figure 2.16). We assign function to genes of the *A. niger* gentisate cluster based on a combination of evidence, including sequence similarity to functionally characterized genes, protein domain analysis, transcription upregulation upon growth on gentisate, impaired growth of mutants on gentisate, accumulation of predicted intermediates by pathway mutants, and functional complementation by characterized genes. We identified *gtaR* (NRRL3_05233) as encoding the transcription regulator of gentisate catabolic genes. The catabolism of gentisate in *A. niger* follows similar steps to that of *Ralstonia* sp. (Zhou *et al.*, 2001). The clustered genes involved are *gdoA* (NRRL3_05236), encoding gentisate 1,2-dioxygenase in the conversion of gentisate to maleylpyruvate, *miaA* (NRRL3_05235), encoding a protein involved in the conversion of maleylpyruvate to fumarylpyruvate, and *fphA*, encoding fumarylpyruvate hydrolase in the conversion of fumarylpyruvate to fumarate and pyruvate.

That the growth defect of $\Delta miaA$ ($\Delta NRRL3_05235$) on gentisate can be rescued by the bacterial maleylpyruvate isomerase gene or its *A. niger* orthologues (Figure 2.10) is a clear indication that *miaA* is essential for the conversion of maleylpyruvate to fumarylpyruvate. The straightforward interpretation of this result and other evidence provided in this study is that *miaA* encodes a maleylpyruvate isomerase despite the lack of sequence similarity with known maleylpyruvate isomerases. Attempts to heterologously express *miaA* in *Escherichia coli* and characterize the recombinant enzyme were unsuccessful, as the protein accumulated in inclusion bodies and did not show maleylpyruvate isomerase activity following solubilization.

The three characterized maleylpyruvate isomerases (glutathione-dependent, mycothiol-dependent, and L-cysteine-dependent) have been shown to be phylogenetically unrelated based on sequence comparisons (Jones & Cooper, 1990; Goetz & Harmuth, 1992; Zhou *et al.*, 2001; Hagedorn *et al.*, 1985; Shen *et al.*, 2005; Feng *et al.*, 2006; Liu & Zhou, 2012, Table 2.2).

To determine whether these analogous proteins share common structural features, we used AlphaFold-predicted protein structures and the crystal structure of glutathione-dependent maleylpyruvate isomerase for comparison. Figure S2.11 shows the AlphaFold and crystal structures of maleylpyruvate isomerases and the alignment of similar proteins. The crystal structure of the glutathione-dependent maleylpyruvate isomerase of *Ralstonia* sp. (Marsh *et al.*, 2008) is highly similar in structure to its *A. niger* orthologues MpiA and MpiB. The structure of the glutathione-dependent maleylpyruvate isomerase is distinct from that of the mycothiol-dependent and L-cysteine-dependent maleylpyruvate isomerases. Despite sequence dissimilarity, the L-cysteine-dependent maleylpyruvate isomerase of *Paenibacillus* sp. shares a similar structure over part of the length of the larger mycothiol-dependent maleylpyruvate isomerase of *C. glutamicum* (Figure S2.12). Hence, these two proteins represent another example of convergent evolution of protein structure. The protein structure comparisons also show that MiaA does not display structural similarity with any of the maleylpyruvate isomerases.

A search of the protein structure database with the AlphaFold-predicted MiaA uncovers an orthologue in the CENP-V/GFA domain-containing protein of *Pseudomonas aeruginosa* (PDB ID: 8AJQ) (Figure S2.13). The GFA domain was originally described for the protein glutathione-dependent formaldehyde-activating enzyme of *Paracoccus denitrificans*, involved in formaldehyde detoxification (Goenrich *et al.*, 2002). The GFA domain is conserved and is found in the human centromere protein CENP-V (Tadeu *et al.*, 2008). Hence, the updated domain name is CENP-V/GFA. A more detailed analysis shows that the Gfa protein of *P. denitrificans* is a carrier of glutathione and does not engage in catalysis (Hopkinson *et al.*, 2015). The *A. niger* genome contains nine genes encoding proteins with the CENP-V/GFA domain (NRRL3_00287, NRRL3_03872, NRRL3_03943, NRRL3_05235, NRRL3_06178, NRRL3_08977, NRRL3_09465, NRRL3_10034, NRRL3_11119). They are small proteins, with lengths ranging from 127 to 186 amino acids, containing only the CENP-V/GFA domain with its seven-cysteine

motif (Prosite PS51891). These observations suggest that the CENP-V/GFA domain-containing proteins in *A. niger* are glutathione carriers for enzymes that require glutathione as a cofactor to perform a variety of reactions. While these observations do not exclude the possibility that MiaA is a maleylpyruvate isomerase, they suggest that MiaA is a glutathione carrier that facilitates the conversion of maleylpyruvate to fumarylpyruvate in gentisate catabolism.

The mechanism by which MiaA mediates the conversion of maleylpyruvate to fumarylpyruvate in gentisate catabolism is unclear. We have ruled out the involvement of MpiA and MpiB, the orthologues of bacterial glutathione-dependent maleylpyruvate isomerase, as $\Delta\text{mpiA}/\text{mpiB}$ has no adverse effect on gentisate utilization (Figure 2.3). One possibility is that the fumarylpyruvate hydrolase FphA is involved in the isomerization of maleylpyruvate with the help of MiaA. However, LC-MS shows the accumulation in ΔfphA of a compound with the same molecular mass, but different retention time as maleylpyruvate, suggesting that fumarylpyruvate can be formed in the absence of FphA. Hence, if the isomerization of maleylpyruvate involving MiaA requires another protein, we currently do not have a candidate for such a protein. The lack of a likely maleylpyruvate isomerase in the gentisate catabolic pathway leads to the possibility that MiaA provides the suitable condition for the isomerization of maleylpyruvate to fumarylpyruvate to occur spontaneously. Regardless of the mechanism, MiaA represents a new protein mediating the isomerization of maleylpyruvate to fumarylpyruvate, in addition to the three phylogenetically unrelated thiol-dependent maleylpyruvate isomerases.

In bacteria and fungi, gentisate is an intermediate in the degradation of aromatic precursors, including salicylic acid (2-hydroxybenzoic acid, 2-HB) and 3-hydroxybenzoic acid (3-HB). Degradation of aromatics in fungi, as in bacteria, can involve complex and/or multiple pathways (Lubbers *et al.*, 2019). For example, in the yeast *C. parapsilosis*, the genes predicted to be involved in gentisate catalysis are responsible for the degradation of 3-HB, and these genes are located in the gene cluster that includes the gene for the conversion of 3-HB to gentisate (Liu & Zhou, 2012). While degradation of 3-HB in *A. terreus* includes gentisate as an intermediate (Martins *et al.*, 2021), in the white-rot fungus *Phanerochaete chrysosporium*, 3-HB is degraded through the 3,4-dihydroxybenzoate branch of the β -ketoadipate pathway (Matsuzaki & Wariishi, 2005). In addition, degradation of 2-HB in *A. niger* utilizes the catechol branch of the β -ketoadipate pathway

(Lubbers *et al.*, 2021). We tested the growth of the gentisate pathway mutants on 2-HB, 3-HB and related aromatics (Figure S2.14). Growth on 2-HB, 3-HB and other tested aromatics was not affected by the gentisate pathway mutations. These results support the idea that in *A. niger*, gentisate is either not an intermediate in the degradation of 2-HB and 3-HB or that these aromatics can be degraded by other pathways besides the gentisate degradative pathway. This study, therefore, proposes the pathway for gentisate catabolism in *A. niger* as shown here (Figure 2.17).

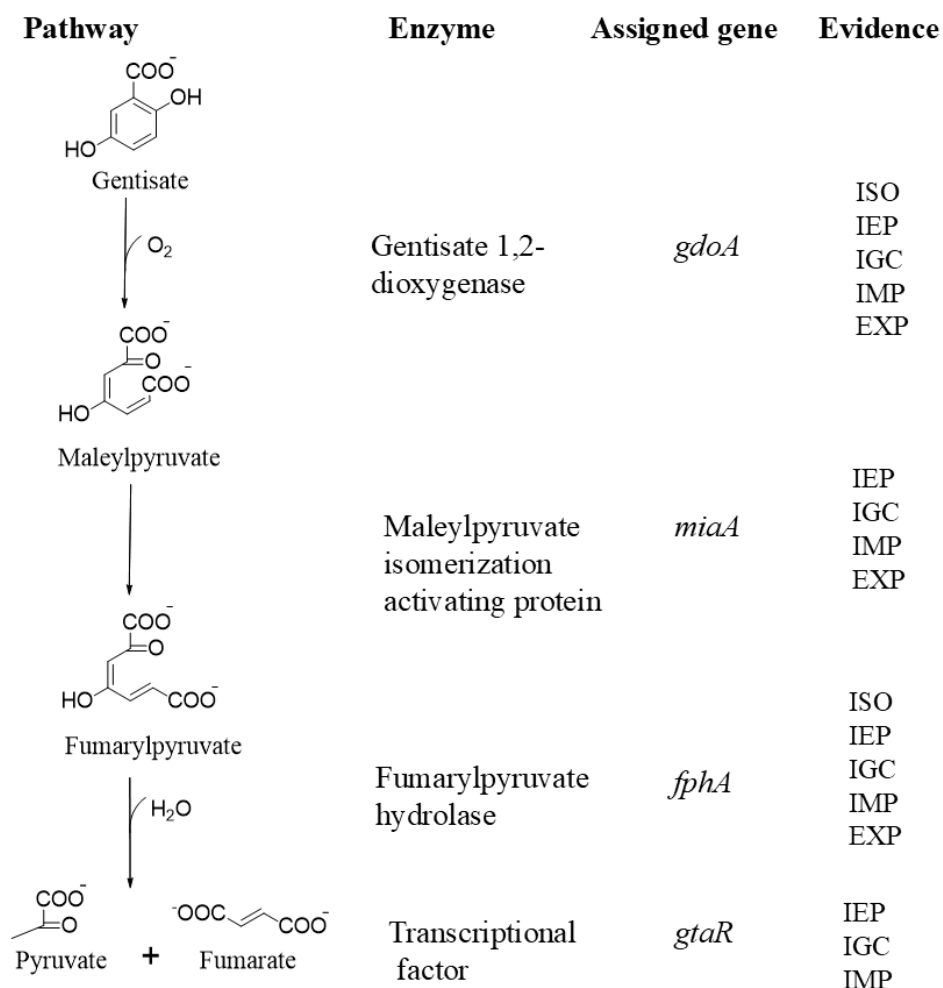


Figure 2.17 Pathway for the catabolism of gentisate in *A. niger*. The genes were assigned based on evidence provided in this study, as denoted by the following codes: ISO, inferred from orthology; IEP, inferred from expression pattern; IMP, inferred from mutant phenotype; IGC, inferred from genomic context; and EXP, inferred from experiment.

2.5 Materials and Methods

2.5.1 Chemicals

Gentisate, fructose and other aromatic compounds used in this study were bought from Sigma-Aldrich (Oakville, ON, Canada). All chemicals were of the highest commercially available purity and were used as received without further purification.

2.5.2 Strains, media and conditions of growth

All strains used in this study are listed in Table 2.3. The parental strain of *A. niger* used in this study was CBS 138852 (NRRL3 *cspA1* Δ *pyrG* Δ *kusA*) (Alazi *et al.*, 2016), a derivative of strain N593 that is auxotrophic for uridine (Goosen *et al.*, 1987) and carries a *kusA* gene deletion (Alazi *et al.*, 2016), which enhances homologous recombination frequency (Meyer *et al.*, 2010). Following protoplastation of the parental strain, transformants were cultured on selective regeneration medium plates for 5 days at 30°C. For genomic DNA extraction and PCR screening, spores from transformants were inoculated into 200 μ L of complete medium (minimal medium supplemented with 10 mM uridine, 1 g/L casamino acids, and 5 g/L yeast extract) and incubated at 30°C for 17 hours. For the growth test of mutant strains, 2 μ L of saline/Tween containing 1000 freshly harvested spores were spotted on minimal medium plates with 0.5% fructose or different aromatic compounds as the sole source of carbon (Hill & Kafer, 2001). All cloned plasmids were propagated and maintained using *Escherichia coli* strain DH5 α . For metabolite analysis by mass spectrometry, spores (2×10^6 spores/mL) of the parent strain as well as mutant strains were inoculated into 200 mL of complete medium with 1% fructose in a rotatory shaker at 220 rpm, 30°C. Following overnight incubation, freshly germinated mycelia were harvested on Miracloth (Merck KgaA, Darmstadt, Germany) and washed with sterile double-distilled water (ddH₂O). Equal amounts of dried mycelia (250 mg) were then transferred to 25 mL of minimal medium with 25 mM gentisate or 25 mM fructose as sources of carbon. The culture was incubated at 30°C and shaken at 220 rpm for up to 48 hours.

Table 2.4 *Aspergillus niger* strains used in this study

Strain	Genotype	Source
N402	<i>cspA1</i> , <i>cspB</i> , derivative of N400 (NRRL3)	(Bos <i>et al.</i> , 1988; Demirci <i>et al.</i> , 2021)
CBS 138852	N593 (ATCC 64973) $\Delta kusA$	(Alazi <i>et al.</i> , 2016)
IBO_1	CBS 138852 $\Delta NRRL3_05234-05237$	This study
IBO_2	CBS 138852 $\Delta NRRL3_10244-10250$	This study
IBO_3	CBS 138852 $\Delta gtaR$ (NRRL3_05233)	This study
IBO_4	CBS 138852 $\Delta NRRL3_05234$	This study
IBO_5	CBS 138852 $\Delta miaA$ (NRRL3_05235)	This study
IBO_6	CBS 138852 $\Delta gdoA$ (NRRL3_05236)	This study
IBO_7	CBS 138852 $\Delta fphA$ (NRRL3_05237)	This study
IBO_8	CBS 138852 $\Delta mpiA$ (NRRL3_04710)	This study
IBO_9	CBS 138852 $\Delta mpiB$ (NRRL3_09971)	This study
IBO_10	CBS 138852 $\Delta mpiA/\Delta mpiB$	This study
IBO_11	CBS 138852 $\Delta miaA::\Delta nagL$	This study
IBO_12	CBS 138852 $\Delta miaA::\Delta mpiA$	This study
IBO_13	CBS 138852 $\Delta miaA::\Delta mpiB$	This study
IBO_14	CBS138852 $\Delta miaA::nagL \Delta fphA$	This study
IBO_15	CBS138852 $\Delta miaA::\Delta mpiA \Delta fphA$	This study
IBO_16	CBS138852 $\Delta miaA::\Delta mpiB \Delta fphA$	This study

2.5.3 Bioreactor cultivation

Bioreactor batch cultivations with *Aspergillus niger* were performed as previously described (Jørgensen *et al.*, 2010; Niu *et al.*, 2017), using 0.75% gentisate as the sole carbon source. RNA extraction for transcriptome analysis was performed using the MagMax Plant RNA Isolation Kit. The transcriptome analysis data from bioreactor cultivations of *A. niger* grown on 0,75 % fructose (Niu *et al.*, 2017) were used as a reference.

2.5.4 RNA sequencing and transcriptome analysis

The RNA-seq data from bioreactor samples of the *A. niger* strain M234.1 grown on gentisate are available in the Sequence Read Archive under accession SRP572550. The RNA-seq data for *A. niger* grown on fructose were previously deposited in the Sequence Read Archive under accession SRP078485 (Niu *et al.*, 2017).

The sequencing process was conducted using Illumina HiSeq 4000 paired-end sequencing technology at the Centre d'expertise et de services Génome Québec, with a read length of 100. The bbdut.sh script in the BBMap package (Bushnell, 2014) was used to pre-process the raw RNA-seq reads to remove sequencing adapters and reads derived from phix and ribosomal RNA. The data were then aligned to the genome of NRRL3 using RSubread software (Liao *et al.*, 2019).

2.5.5 Construction of *A. niger* mutant strains using CRISPR/Cas9

In this study, the previously designed ANEp8-Cas9-LIC plasmid was used to generate mutant strains (Song *et al.*, 2018). CRISPR/Cas9 guide RNA sequences were predicted using Geneious R9.1 (<http://www.geneious.com>, (Kearse *et al.*, 2012) and constructed as previously described with minor modifications (Song *et al.*, 2018). Successful guide RNA expression constructs were cloned into ANEp8-Cas9-LIC plasmid by ligation-independent cloning, and the resulting construct was subsequently transformed into *Escherichia coli* DH5 α as previously described (Song *et al.*, 2018). The primers and sequences used are listed in the legends of the verification figures as previously shown in Figures S2.1-S2.10. Single-stranded 60 or 90-base gene-editing oligonucleotides designed to serve as a rescue template were cloned into *A. niger* in addition to the CRISPR plasmid DNA by co-transformation. These oligonucleotides were used to repair the double-stranded breaks created in the target genes by Cas9 nuclease, as previously shown in Figures S2.1-S2.10.

2.5.6 Transformation of *A. niger* and verification of mutant strains

A. niger protoplastation and transformation were performed as previously described with minor modifications (Debets & Bos, 1986; Meyer *et al.*, 2010). One microgram (1 μ g) of CRISPR

plasmid DNA and 1 nmol of the corresponding rescue template, together with 200 μ L of the protoplast (1×10^7 protoplasts/mL), were used for each transformation. Transformed protoplasts were grown for up to 5 days at 30°C on selective regeneration medium (SRM) plates lacking uracil or uridine. Putative independent transformants for each gene were isolated and purified for growth test analysis. Mutations in the transformants were confirmed using the knockout screening primers by PCR amplification of sequences surrounding the deletion site. Agarose gel electrophoresis was used to visualize amplified DNA fragments (Figures S2.1 – S2.10).

2.5.7 Construction of gene replacement cassettes for $\Delta NRRL3_05235$ in *A. niger*

Gene replacement cassettes for the $\Delta NRRL3_05235$ locus were constructed. Primers were designed to amplify the previously characterized bacterial maleylpyruvate isomerase (*nagL*) gene from *Ralstonia* sp. strain U2 (UniProt ID O86043). The DNA fragments coding for the investigated proteins were inserted into an empty pJET plasmid (CloneJET PCR cloning kit, Thermo Scientific) along with the promoter and terminator sequences of *NRRL3_05235* using the seamless ligation-independent cloning technique (SLiCE) (Zhang *et al.*, 2014). Additionally, two bacterial maleylpyruvate isomerase orthologues in *Aspergillus niger*, *NRRL3_09971* and *NRRL3_04710*, were also cloned into pJET plasmids with the promoter and the terminator sequences of *NRRL3_05235*. The resulting constructs were transformed in *A. niger* to replace the disrupted $\Delta NRRL3_05235$ locus (Table 2.3). Similarly, replacement cassettes as described above were constructed and cloned into the *NRRL3_05235* locus, but in the $\Delta NRRL3_05237$ strain.

2.5.8 Metabolite analysis from mutant strains using liquid chromatography-mass spectrometry (LC-MS)

Preliminary cultures of parental and mutant strains were carried out in a 1L flask containing 100 mL complete medium with 1% fructose and inoculated with 2×10^8 freshly harvested spores. The precultures were incubated at 30 °C, 220 rpm for 16-18 hours. Following the transfer of duplicate cultures of mycelia into minimal medium containing gentisate, samples of the cultures were collected at different time points in 1.5 mL microcentrifuge tubes and centrifuged at 13,000 $\times g$ for 25 minutes. Supernatants were transferred to fresh tubes, and two volumes of cold methanol were added. The mixtures were incubated on ice for 10 minutes and

centrifuged at 13,000 $\times g$ for 25 minutes to precipitate protein. Finally, supernatants were transferred to new tubes, and equal volumes of 0.1% formic acid were added. The detection of accumulated metabolites was analyzed using LC-MS. A 10 μ L aliquot was analyzed by liquid chromatography-mass spectrometry using an Agilent 1290 Infinity II HPLC system (Agilent Technologies, Santa Clara, CA, USA) connected in-line to an Agilent 6560 Ion mobility QTOF mass spectrometer equipped with a Jetstream electrospray source. Chromatography separation of sample components was done using a Synergy Hydro-RP 150 $\times$ 2.0 mm, 4 μ m column (Phenomenex, Torrance, CA, USA). The solvents used to generate the gradient during reversed-phase separation were 0.1 % formic acid in water for Solvent A and 0.1% formic acid in acetonitrile for Solvent B. The solvent flow rate was 300 μ L/min. The gradient started at 2% B, increased to 30% B in 5 min, then increased to 80% B in 3 min, was kept at 100% B for 1.5 min, decreased to 2% B in 0.1 min and held at 2% B for 4 min. Full-range MS scans from 50 to 1100 m/z were acquired at 2 Hz in negative polarity. The relative abundance assessment of the compound of interest was performed by comparing extracted ion chromatograms (EICs) and determining peak areas. The Agilent Mass Hunter qualitative software was used to generate EICs using a 10-ppm extraction window of the theoretical monoisotopic singly charged m/z value for each compound.

2.5.9 Generation of maleylpyruvate

To generate maleylpyruvate *in vitro*, previously purified gentisate 1,2-dioxygenase (NRRL3_05236) was incubated with gentisate as the substrate in 50 mM Tris-HCl buffer (pH 7.6) for 10 minutes at room temperature. Following incubation, the reaction mixture was analyzed by UV-Vis spectrophotometry to detect product formation. The presence of maleylpyruvate was inferred from an absorption maximum at 330 nm, consistent with previously published reports identifying this wavelength as characteristic of maleylpyruvate (Fang *et al.*, 2011).

2.5.10 Protein modelling

NRRL3 protein 3D models were generated using AlphaFold (Jumper *et al.*, 2021) and obtained from either the RCSB Protein Data Bank (<https://www.rcsb.org>) (Berman *et al.*, 2000) or the AlphaFold Protein Structure database (<https://alphafold.ebi.ac.uk/>) (Varadi *et al.*, 2022; Varadi *et al.*, 2024). Predicted structures were compared to functionally characterized and experimentally

determined protein structures available from the Protein Data Bank. When the experimentally determined protein structures included more than one protein monomer, the structure of a single AlphaFold-predicted monomer was used after confirmation of close alignment with the experimentally determined structure. Alignments were performed using PyMOL v. 3.0.0 (Schrodinger, 2010).

2.6 Data availability

All data are contained within the manuscript.

2.7 Funding and additional information

This work was supported by the Biocatalysis Strategic Network of the Natural Sciences and Engineering Research Council of Canada.

2.8 Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

2.9 Supplementary Figures

Figure S2.1 – S2.10 PCR verification of deletions in the gentisate pathway genes.

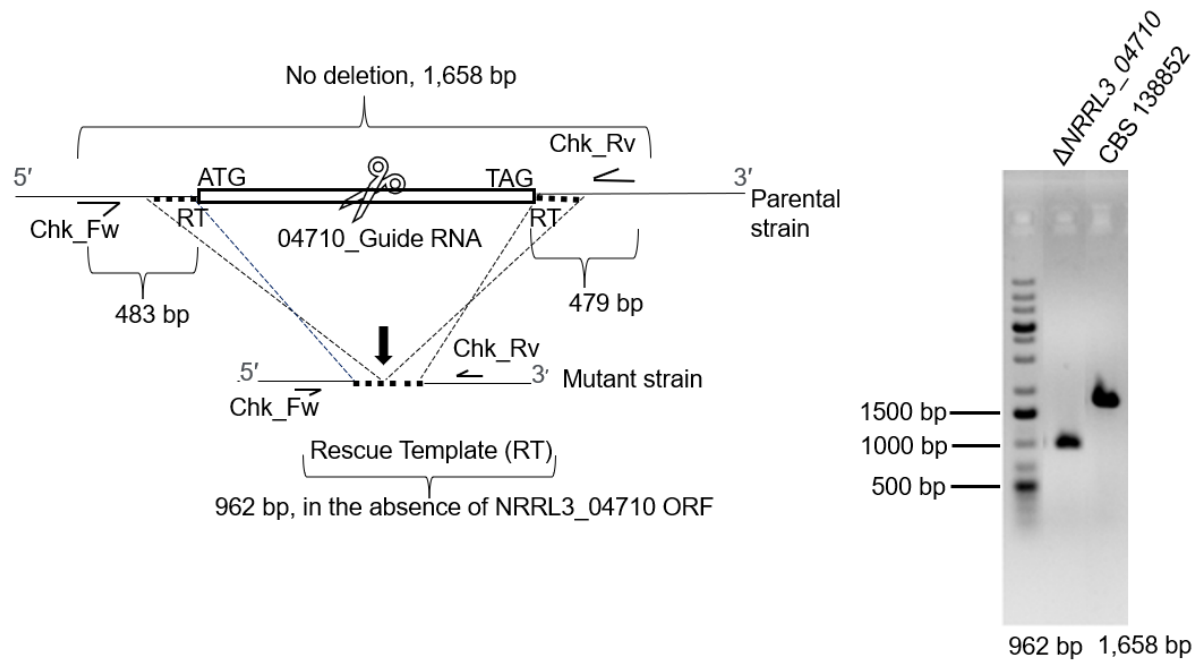


Figure S2.1 PCR verification of *NRRL3_04710* (Δ *mpiA*). The target sequence for the CRISPR/Cas9 gRNA was GATAACGTGGAGGATCAGGT. The rescue template oligonucleotide-sequence-ACACAAGTTTAAAGTCGTCCGGCTAAGAAG-CTCTGGCCATTATGCGGGGTGTCCCAGTTG with complementary sequences on either side of the intended deletion was used to repair the double-stranded break. Primers flanking the deletion (04710Fw: GAGGCTTTGAGGCGCTTGTG and 04710Rv: CTTGCATGGAAGCCCGAAGTC) were used for PCR verification, resulting in a 962 bp fragment in the mutant as compared to the 1,658 bp in the parental strain

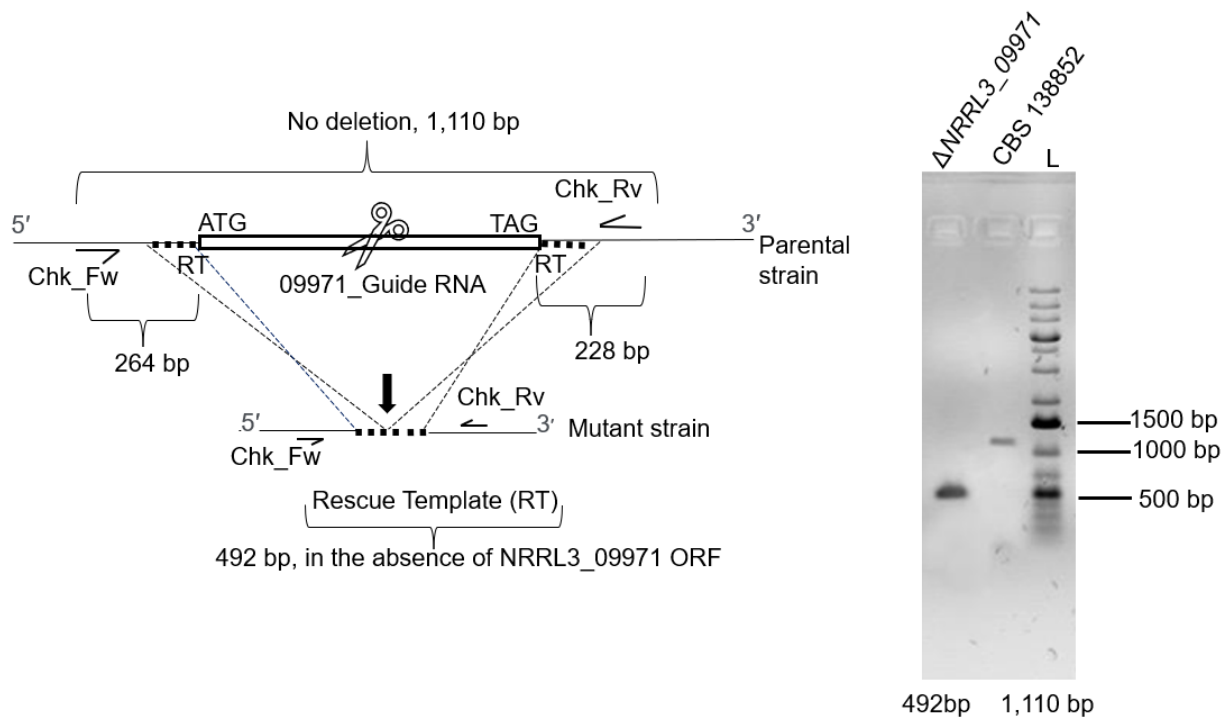


Figure S2.2 PCR verification of *NRRL3_09971* (Δ *mpiB*). The target sequence for the CRISPR/Cas9 gRNA was CTCGGTGTGGACAGGGAAAC. The rescue template oligonucleotide sequence-ATGTCCGAACAAACCATAACCCTGCACACCTACTTCGCTCCTCG-TGATAGGCAGTAAGGCAATGGTGGGATGAAATAATGGAAGGTTGG with complementary sequences on either side of the intended deletion was used to repair the double-stranded break. Primers flanking the deletion (09971Fw: GCAGGTACTGTACCACACACAC and 09971Rv: GGAGTCACGGAGTCAAGTCAAAC) were used for PCR verification, resulting in a 492 bp fragment in the mutant compared to the 1,110 bp in the parental strain.

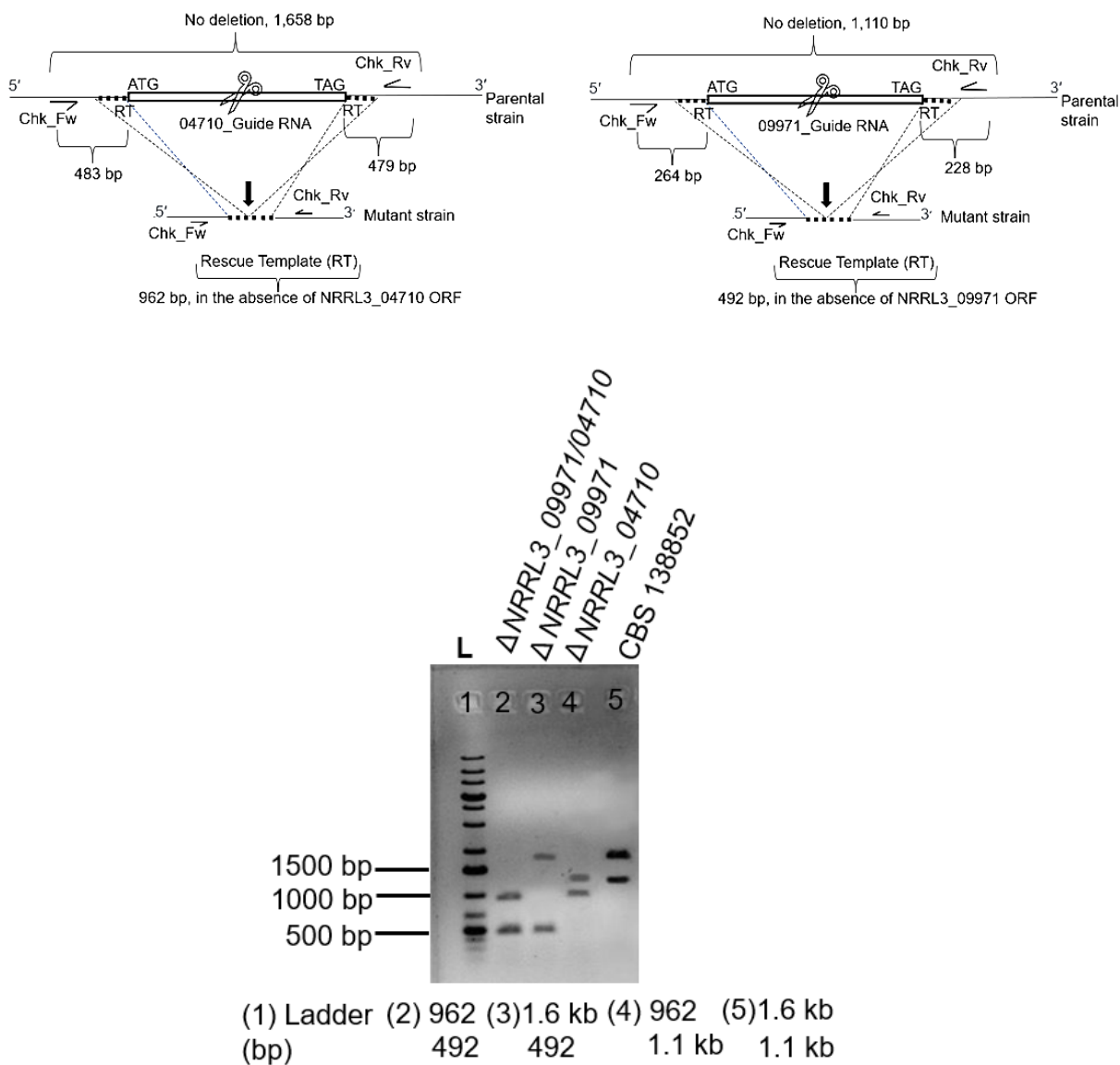


Figure S2.3 PCR verification of $\Delta NRRL3_04710$, $\Delta NRRL3_09971$ and $\Delta NRRL3_04710/\Delta NRRL3_09971$. The target sequences for the CRISPR/Cas9 gRNAs and the rescue template oligonucleotide sequences for the deletions of *NRRL3_04710* and *NRRL3_09971* were the same as in Figure S2.1 and Figure S2.2, respectively. Primers flanking the deletion (04710Fw: GAGGCTTTGAGGCGCTTGTG and 04710Rv: CTTGCATGGAAGCCCGAAGTC: 09971Fw: GCAGGTACTGTACCACACACAC and 09971Rv: GGAGTCACGGAGTCAAGTCAAAC) were used for PCR verification. Expected band sizes for mutant and parent strains are shown below each PCR product.

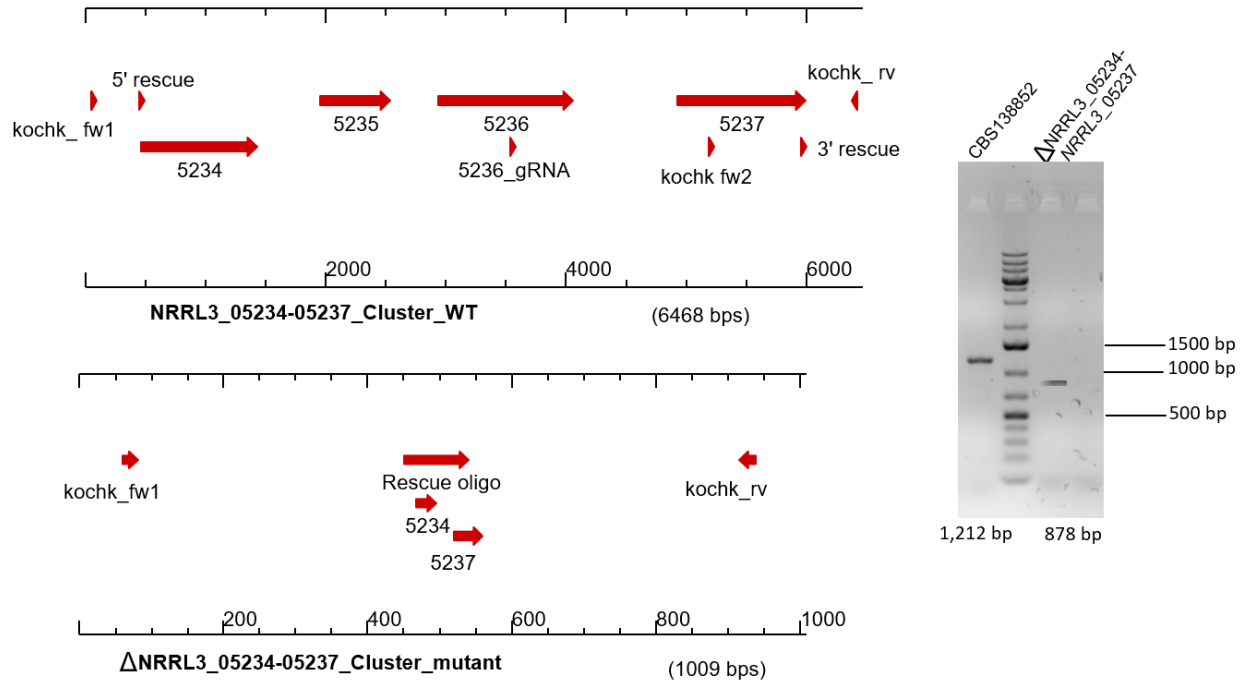


Figure S2.4 PCR verification of Δ NRRL3_05234-05237. The target sequence for the CRISPR/Cas9 gRNA was CGACGGATACATCGGCACCT. The rescue template oligonucleotide-sequence-GACTTGCCCTGCCCATCATGGTCCAAGCAAAAGCCTTCATTGTCCC-AGGGGCTGGGTGCGACGAGGAACGTTATGGTTTTTGAGTAGGGAG with complementary sequences on either side of the intended deletion was used to repair the double-stranded break. Primers flanking the deletion (Δ NRRL3_05234-05237 Check Fw1: CCTGGAGGAAGACCTACCTGTG, Δ NRRL3_05234-05237 Check Fw2: GTGCAAGACTCAGTTGGTACCAG and Δ NRRL3_05234-05237 Rv: GATTAAGACGGTGGAGGCGGATAG) were used for PCR verification, resulting in an 878 bp fragment in the mutant compared to the 1,212 bp in the parental strain.

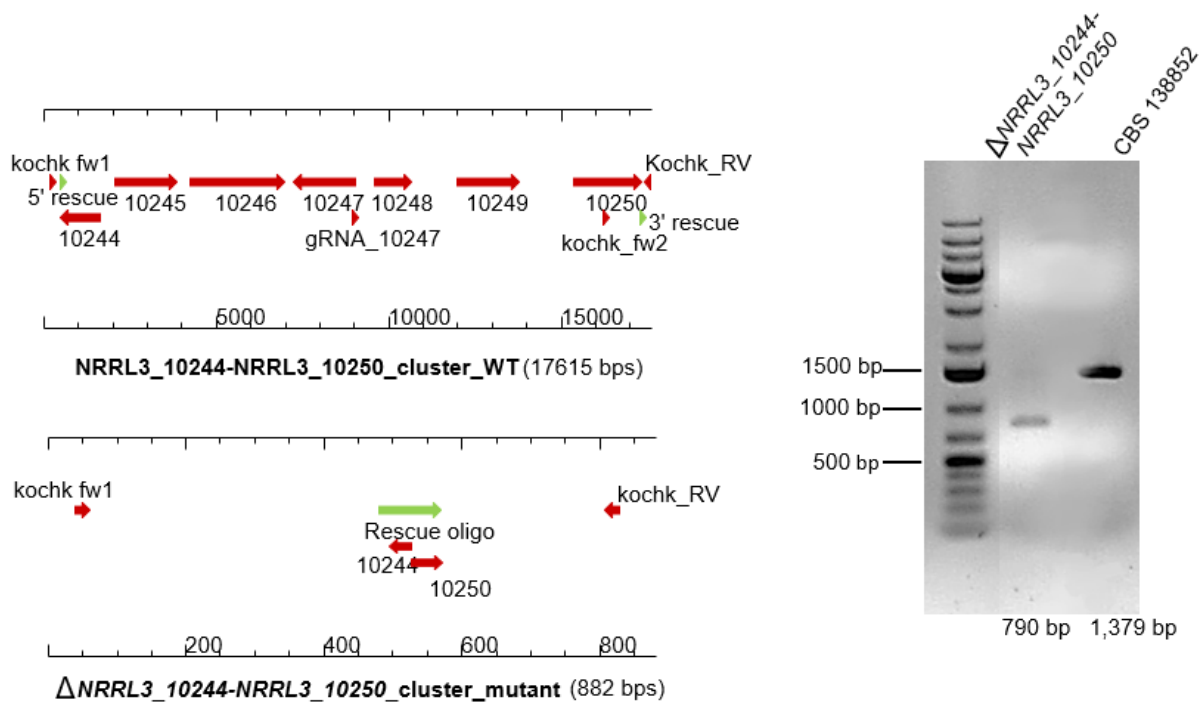


Figure S2.5 PCR verification of Δ NRRL3_10244-NRRL3_10250. The target sequence for the CRISPR/Cas9 gRNA was AACCCTGAATCTGGAGGTCT. The rescue template oligonucleotide sequence- GGCAGTTCAAGGTTCTACTGGGACACCAGCGTCGCCAGATCCGTAT-CTGGGGGTGAAGTTTGGGTTGTGGAATGCTACGAATCCTTATTG with complementary sequences on either side of the intended deletion, was used to repair the double-stranded break. Primers flanking the deletion (Δ NRRL3_10244-NRRL3_10250 Fw1: CACTACTCTCCAGGGCTGGATC: Δ NRRL3_10244-NRRL3_10250 Fw2: CCAGCTAACGCGGTCTAGTTG: Δ NRRL3_10244-NRRL3_10250_Rv: CACTACTCTCCAGGGCTGGATC were used for PCR verification, resulting in a 790 bp fragment in the mutant compared to the 1,379 bp in the parental strain.

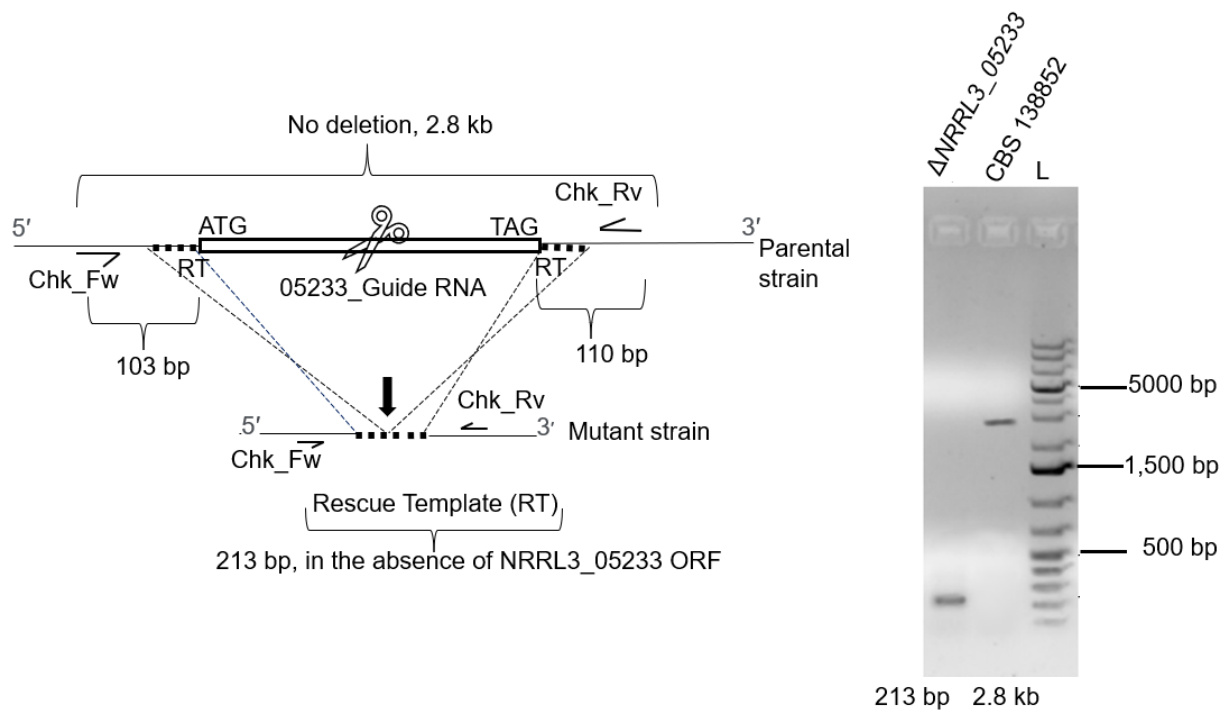


Figure S2.6 PCR verification of NRRL3_05233 (Δ gtaR). The target sequence for the CRISPR/Cas9 gRNA was AAGAAGCGCTCCTTTCAGCG. The rescue template (oligonucleotide-sequence-AGAGTCGAATCCTCACGTACTCACACACTA-CGACTTGGTATATGGAGGTTTGTCACTATA) with complementary sequences on either side of the intended deletion was used to repair the double-stranded break. Primers flanking the deletion (05233Fw: CCGCAAATGCAGGTTGTCGG and 05233Rv: GAACGTGGACAGTCATCCGC) were used for PCR verification, resulting in a 213 bp fragment in the mutant as compared to the 2.8 kb in the parental strain.

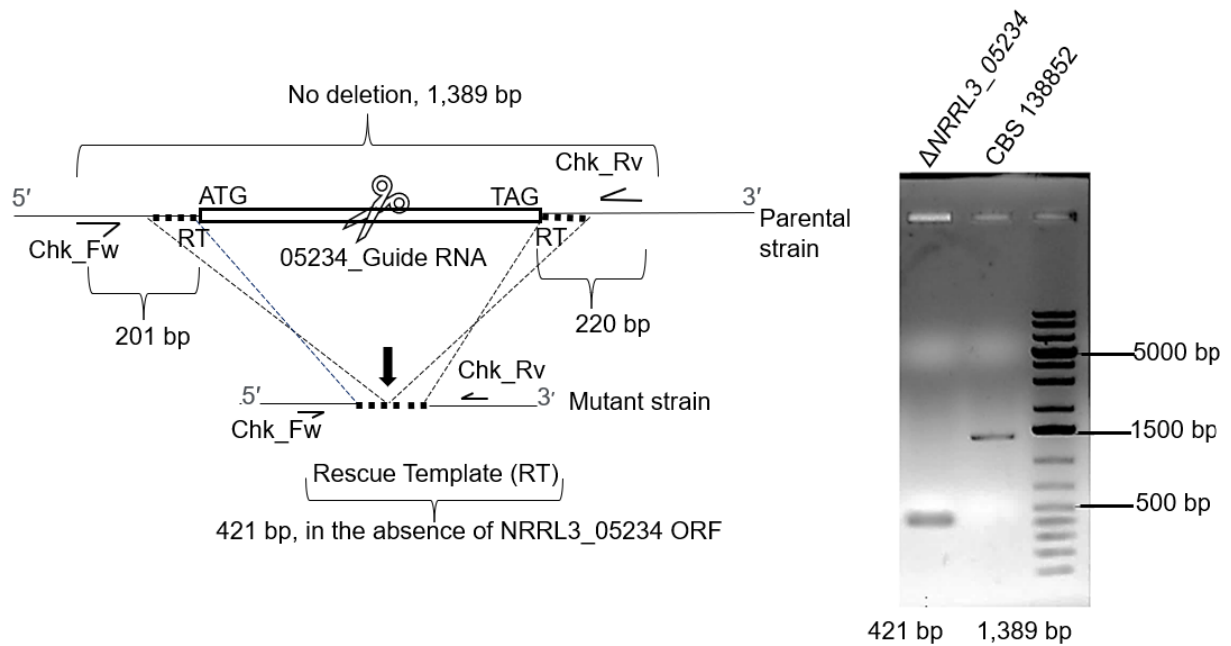


Figure S2.7 PCR verification of Δ NRRL3_05234. The target sequence for the CRISPR/Cas9 gRNA was CGTGTGGCAGAAATTACCCA. The rescue template (oligonucleotide-sequence-CAACTTCCTCCCTCCCTAGACTTGCCTGCC-GTTTATATTATTCTATTGAGATGTTTCAAG) with complementary sequences on either side of the intended deletion was used to repair the double-stranded break. Primers flanking the deletion (05234Fw: CTCTCCGAACATCCCGAGTTC and 05234Rv: GAACTCGGGATGTTTCGGAGAG) were used for PCR verification, resulting in a 421 bp fragment in the mutant as compared to the 1,389 bp in the parental strain

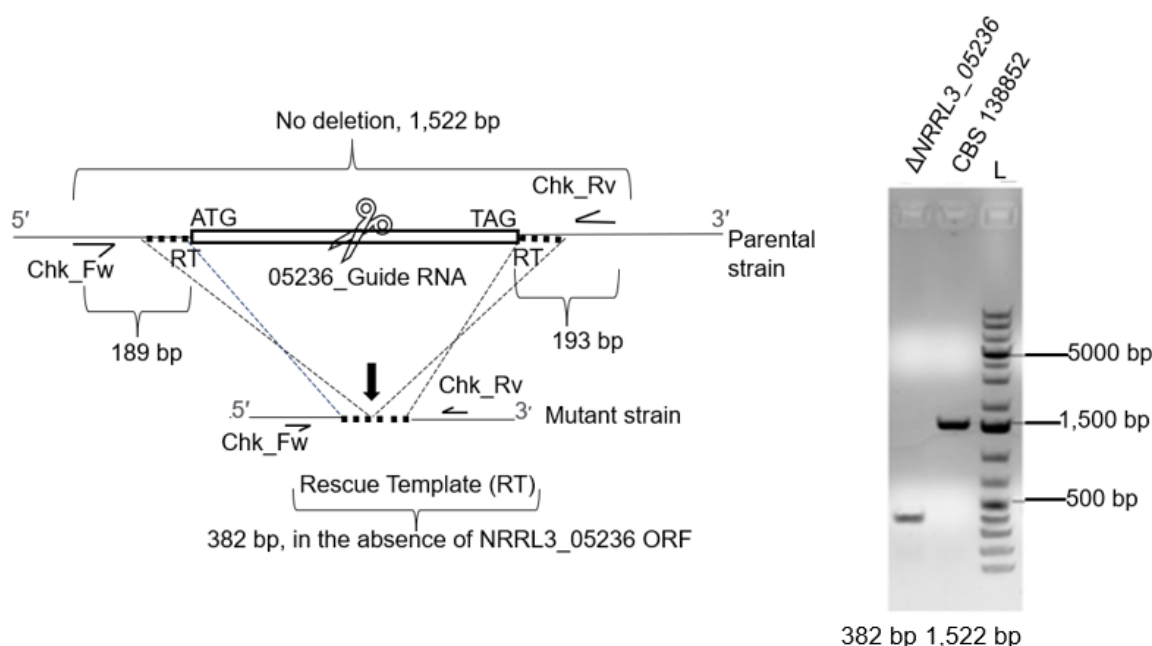


Figure S2.8 PCR verification of *NRRL3_05236* (Δ *gtaA*). The target sequence for the CRISPR/Cas9 gRNA was GTTCCCGGTGAATTCGCAG. The rescue template (oligonucleotide-sequence-CCCAAAGATACCAACGAACAACCAAATAGA-TGGAATGTGATTTGTGCAACCTACTATTAT) with complementary sequences on either side of the intended deletion was used to repair the double-stranded break. Primers flanking the deletion (05236Fw: GCGAGTTCCAGAAGAGCGGG and 05236Rv: GTAGTTGCCACCCTTCTCTCC) were used for PCR verification, resulting in a 382 bp fragment in the mutant as compared to the 1,522 bp in the parental strain.

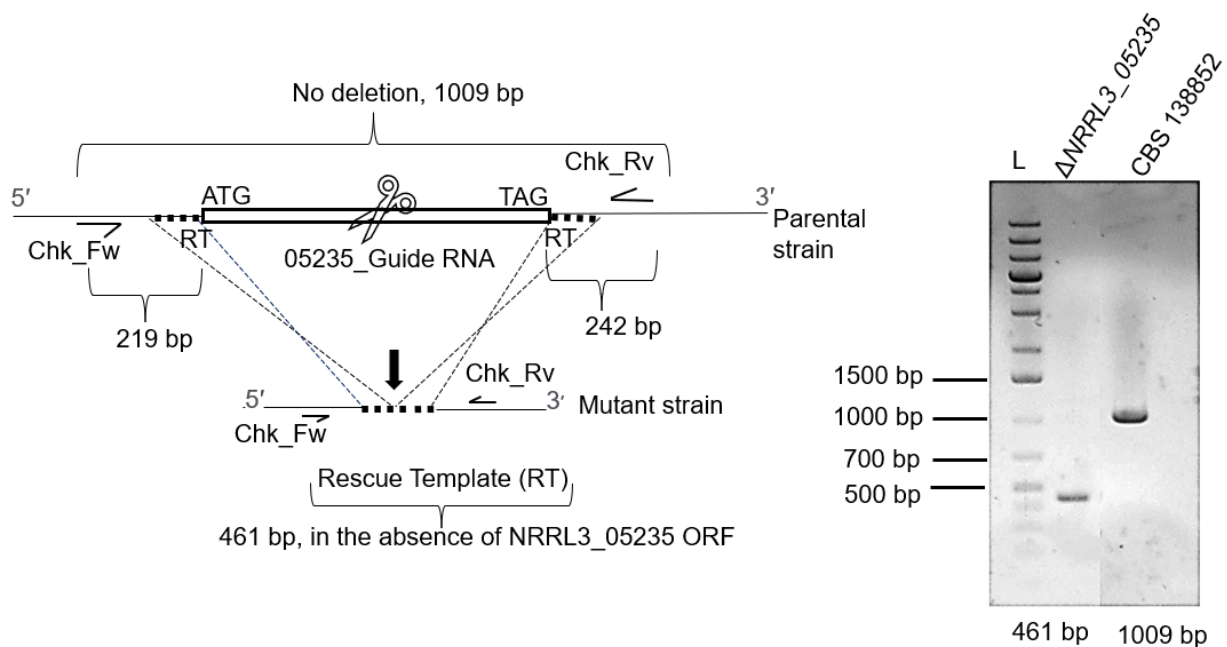


Figure S2.9 PCR verification of *NRRL3_05235* (Δ*miaA*). The target sequence for the CRISPR/Cas9 gRNA was CGACGGATACATCGGCACCT. The rescue template (oligonucleotide-sequence-CGGTCAAAGGAACCTTGGAGACAGCCTACCATCATGAATAAAGCC-GTTTGTGACTATGCCCCGGTCACTGAGACGCTTGTGTATGTAGCC) with complementary sequences on either side of the intended deletion was used to repair the double-stranded break. Primers flanking the deletion (05235Fw: CGAACAAGGACATGATGGGG and 05235Rv: CCAGATGTATTTCACCCCGC) were used for PCR verification, resulting in a 461 bp fragment in the mutant as compared to the 1,009 bp in the parental strain.

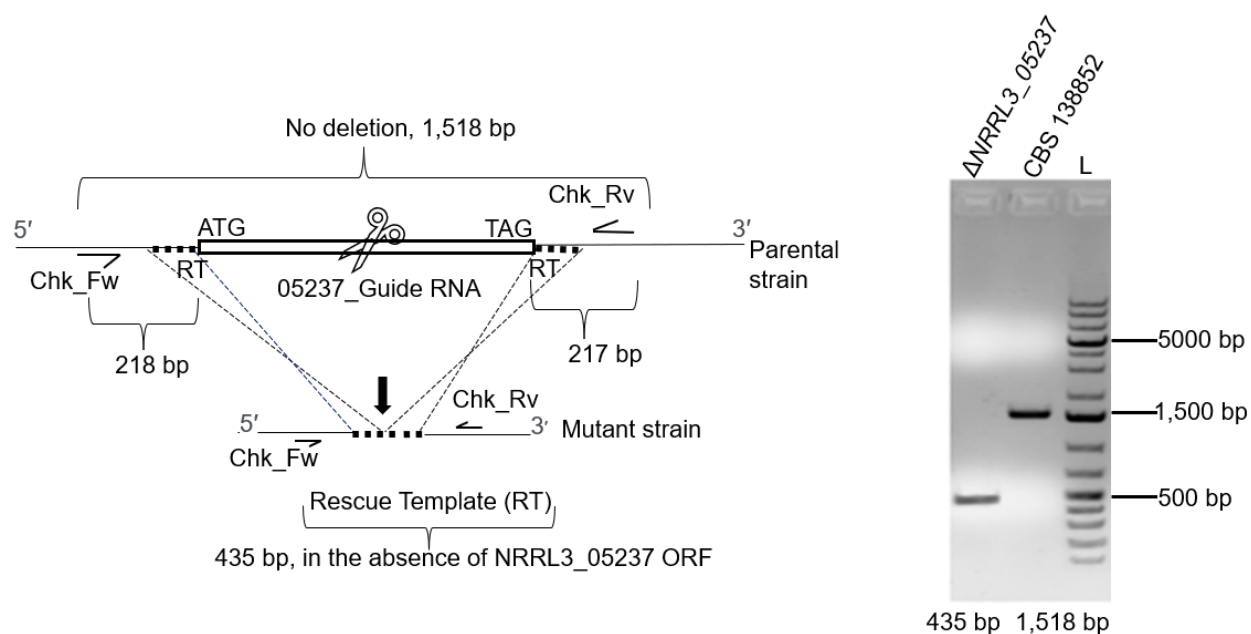


Figure S2.10 PCR verification of NRRL3_05237 (Δ fphA). The target sequence for the CRISPR/Cas9 gRNA was CCGAAGTATCTTAAGGATGG. The rescue template oligonucleotide-sequence-AGCACATTTTCATGCTACAATAAAATATTCA-GTTGCTATTGCCCTGTTTCAGGCTGATTATA, with complementary sequences on either side of the intended deletion, was used to repair the double-stranded break. Primers flanking the deletion (05237Fw: CCGTGTCAATCGGAATGACCG and 05237Rv: CATTGCACTGCACCACAGCAC) were used for PCR verification, resulting in a 435 bp fragment in the mutant as compared to the 1,518 bp in the parental strain.

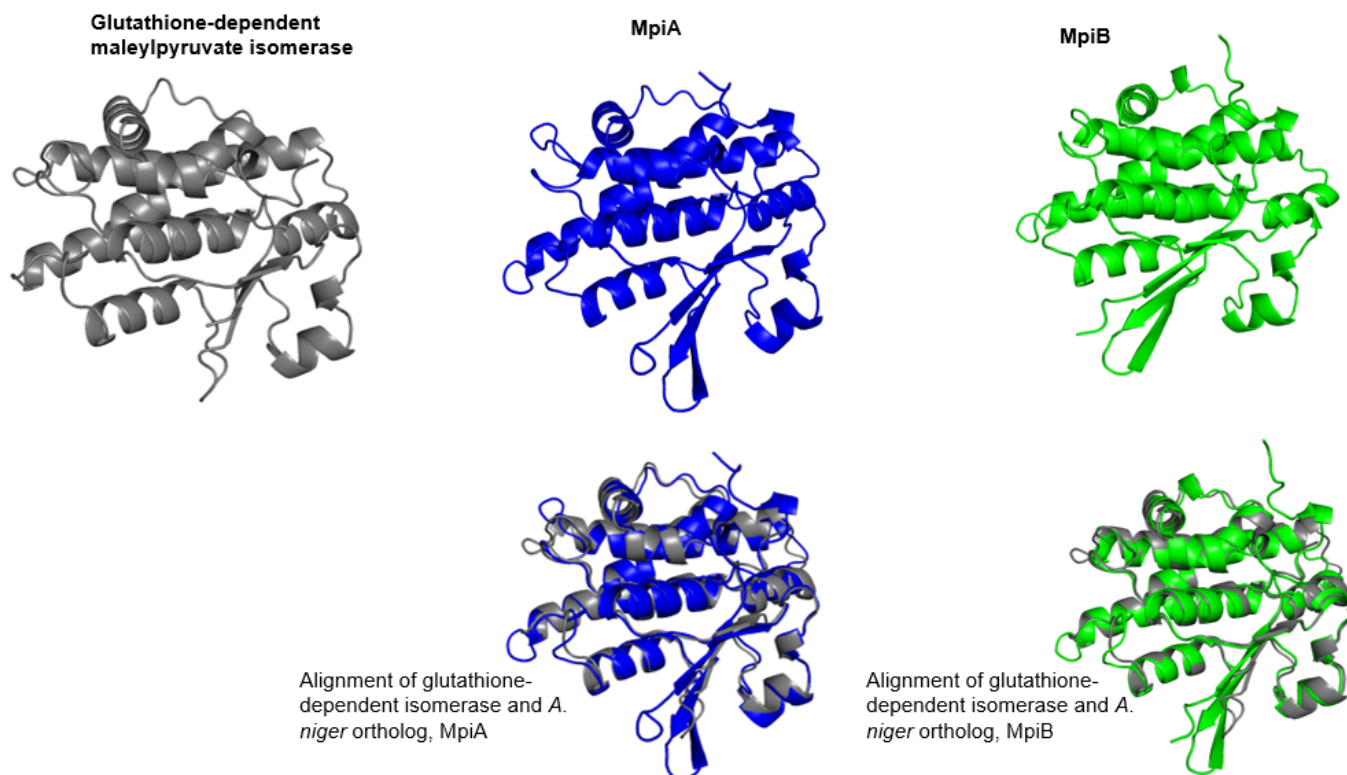


Figure S2.11 Structural alignment between bacterial homolog and AlphaFold-predicted maleylpyruvate isomerase proteins from *A. niger*. Alignment of *A. niger* orthologues MpiA and MpiB with *Ralstonia* sp. MPI (PDB ID: 2JL4), visualized using PyMOL.

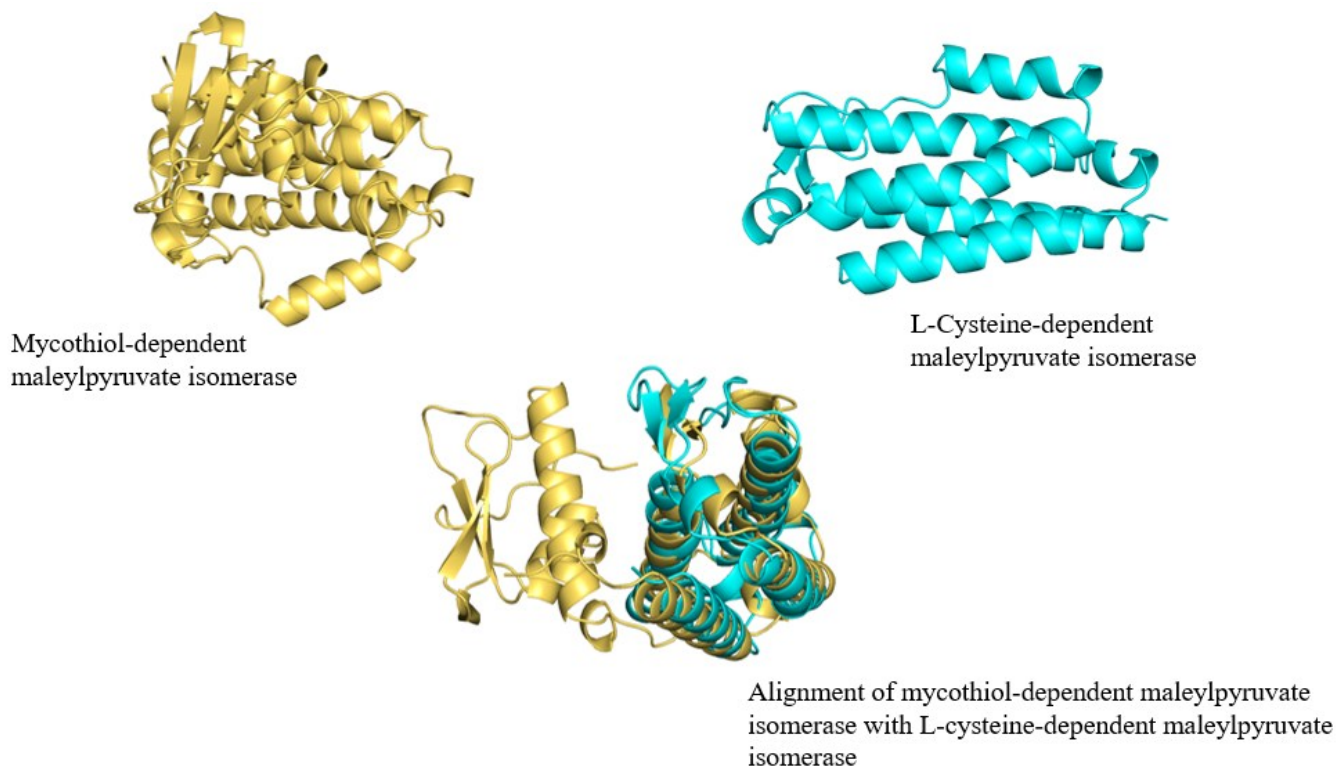


Figure S2.12 Structural alignment of bacterial MPIs. Overlay of mycothiol-dependent maleylpyruvate isomerase (AlphaFold ID; A0A0U4IVI7) and L-cysteine-dependent maleylpyruvate isomerase (AlphaFold ID; H6TG59), visualized using PyMOL

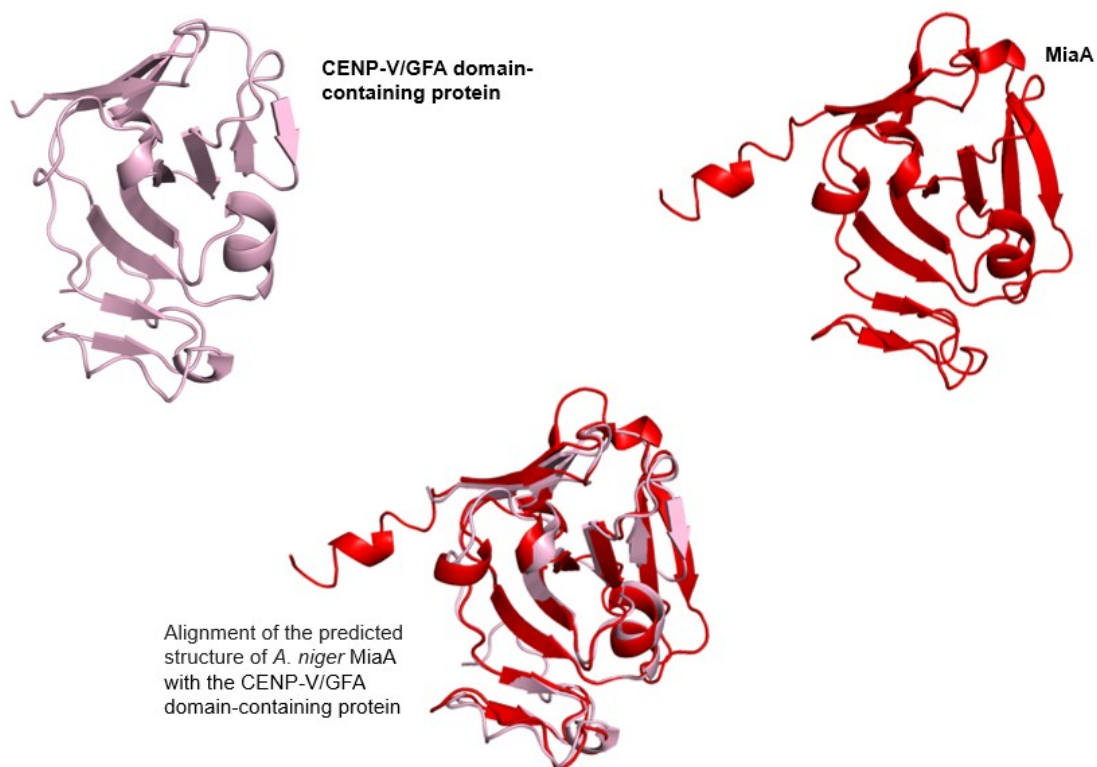


Figure S2.13 Structural alignment of AlphaFold-predicted maleylpyruvate isomerase protein from *A. niger* with bacterial homolog. Comparison of MiaA with *Pseudomonas aeruginosa* CENP-V/GFA domain-containing protein PA2722 (PDB ID: 8AJQ).

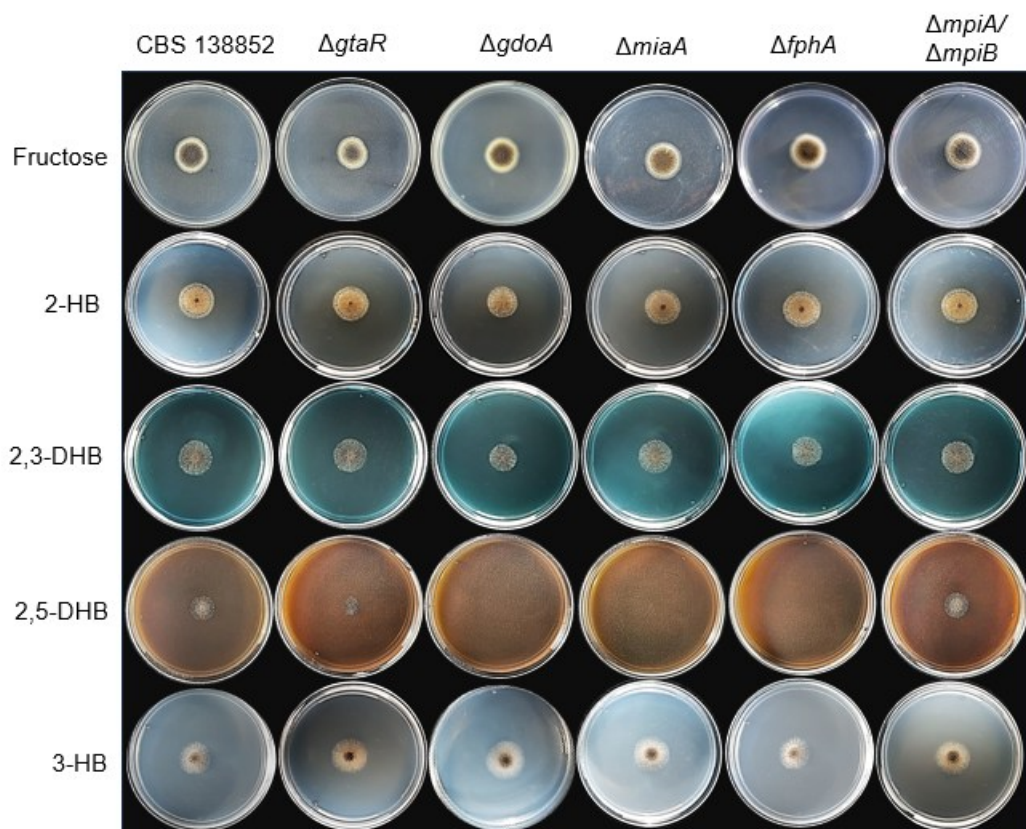


Figure S2.14 Growth phenotype of predicted pathway mutants on various aromatic compounds. Growth of pathway mutants on agar plates containing the following compounds: 2-HB (2-hydroxybenzoic acid, salicylate), 2,3-DHB (2,3-dihydroxybenzoic acid), 2,5-DHB (2,5-dihydroxybenzoic acid, gentisate), and 3-HB (3-hydroxybenzoic acid). Agar plates were incubated at 30°C for 72 hours.

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Chapter 3: Functional characterization of resveratrol catabolic pathway in *Aspergillus niger*

3.1 Abstract

Microorganisms break down plant biomass, releasing aromatic and polyaromatic chemicals; many of these compounds act as plant defense molecules. Resveratrol is a naturally occurring polyphenol found in berries, peanuts, and grapes, and exhibits allelopathic activity by inhibiting the development of competing plants and the growth of pathogens. In bacteria and fungi, resveratrol is metabolized into 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde. Here, we examined the genes involved in the catabolism of resveratrol in the filamentous fungus *Aspergillus niger* by generating pathway mutants, observing their growth phenotype, and analyzing the metabolites that they accumulate in the culture medium. We showed that a previously characterized vanillin dehydrogenase VdhA (NRRL3_03887) is involved in the conversion of both 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde into 3,5-dihydroxybenzoate and 4-hydroxybenzoate, respectively. We further characterized a gene cluster, NRRL3_10244–NRRL3_10248, encoding resveratrol cleavage oxygenase RcoA (NRRL3_10247) that converts resveratrol to 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde, and the genes involved in the catabolism of 3,5-dihydroxybenzoate as follows: 3,5-dihydroxybenzoate 2-monooxygenase DboA (NRRL3_10245) converting 3,5-dihydroxybenzoate to 2,3,5-trihydroxybenzoate; the ring-cleaving 2,3,5-trihydroxybenzoate 1,2-dioxygenase TdoA (NRRL3_10244) converting 2,3,5-trihydroxybenzoate to hydroxymaleylpyruvate / oxaloacetylpyruvate; hydroxymaleylpyruvate / oxaloacetylpyruvate hydrolase OphA (NRRL3_10248); and the regulator of the gene cluster RvtR (NRRL3_10246). These findings provide the first functional evidence for the catabolism of resveratrol and its downstream intermediate 3,5-dihydrobenzoate in *A. niger*. Together with published results on the degradation of 4-hydroxybenzaldehyde, the findings from this study enable us to propose a complete pathway for the catabolism of resveratrol, with *A. niger* genes assigned to each step.

3.2 Introduction

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a stilbene, a member of a subclass of polyphenols characterized by two phenol rings connected by a styrene double bond (Figure 3.1). It is synthesized in plants through the phenylpropanoid pathway and is found predominantly in the skin of grapes, berries, and peanuts (Feng *et al.*, 2022). Resveratrol exists in two isomeric forms: *trans*-resveratrol and *cis*-resveratrol. The *trans*-form is more stable and exhibits extensive biological activities that can benefit both plant and animal systems (Thapa *et al.*, 2019).

Similar to other plant-derived aromatic compounds, resveratrol functions as a defense molecule in plant systems, protecting them against pathogens, pests and environmental stress. The production of resveratrol is typically triggered as part of the plant's innate immune response, especially under conditions of stress such as pathogen attack, UV radiation, or physical damage (Song *et al.*, 2021). Resveratrol acts as a phytoalexin, a substance produced by plants that inhibits the growth of invading microorganisms. The antimicrobial properties of resveratrol are broad-spectrum, demonstrating effectiveness against a wide range of bacterial and fungal pathogens (Zhang *et al.*, 2021). Its mode of action against bacteria includes disrupting the bacterial cell wall and membrane, interfering with the function of essential enzymes, and inhibiting nucleic acid synthesis, leading to impaired cell division and growth (Abedini *et al.*, 2021; Cebrian *et al.*, 2022).

As an antifungal agent, resveratrol inhibits the growth of several plant pathogenic fungi, such as *Botrytis cinerea*, which causes grey mould in grapes and other fruits, and *Plasmopara viticola*, responsible for downy mildew in grapevines (Wu *et al.*, 2023; Rahman *et al.*, 2024). Resveratrol disrupts the integrity of fungal cell walls and membranes by impairing the synthesis of chitin, glucan, and ergosterol (Adrian *et al.*, 1997; Okamoto-Shibayama *et al.*, 2021). Additionally, resveratrol can induce the production of reactive oxygen species, leading to oxidative stress and cellular damage.

The protective role of resveratrol extends beyond direct antimicrobial activity. It strengthens plant cell walls by promoting the cross-linking of cell wall polymers, making it more difficult for pathogens to penetrate and establish infections. It can act as a signalling molecule, activating other defense mechanisms in plants, such as the production of other phytoalexins,

pathogenesis-related proteins, and the reinforcement of plant tissues through lignification (McCalley *et al.*, 2014).

Bacterial and fungal cells can catabolize resveratrol. In the bacterium *Acinetobacter oleivorans* and the endophytic fungus *Phomopsis liquidambari*, resveratrol is catabolized by resveratrol cleavage dioxygenase to 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde (Kurt *et al.*, 2018; Wang *et al.*, 2019). Our understanding of the remaining steps in the catabolic pathway of resveratrol comes from reconstructing pathways for related aromatic metabolites. Based on the observation that 4-hydroxybenzaldehyde in the current *p*-cresol degradation pathway in bacteria is converted to 4-hydroxybenzoate, and its encoding gene is adjacent to the genes of the 3,4-dihydroxybenzoate (protocatechuic acid, 3,4-DHB) degradation pathway (Hopper, 1976; Cronin *et al.*, 1999), Kurt *et al.* (2018) proposed that 4-hydroxybenzaldehyde is converted to 4-hydroxybenzoate by 4-hydroxybenzaldehyde dehydrogenase and subsequently acted upon by 4-hydroxybenzoate hydroxylase to produce 3,4-DHB. These authors (Kurt *et al.*, 2018) further suggested that the second product of resveratrol cleavage oxygenase, 3,5-dihydroxybenzaldehyde, is converted to 3,5-dihydrobenzoic acid (3,5-DHB) by 3,5-dihydroxybenzaldehyde dehydrogenase, with subsequent degradative steps unresolved (Kurt *et al.*, 2018).

Using crude extracts of *Bacillus brevis* grown on 3,5-DHB to analyze downstream intermediates of 3,5-DHB catabolism, Crawford and Perkins (1978) showed that 3,5-DHB is converted to 2,3,5-trihydrobenzoic acid, followed by conversion to hydroxymaleylpyruvic acid, then to pyruvic acid and oxaloacetic acid. By piecing together the catabolic pathways of different metabolites in bacteria and fungi, we hypothesized that resveratrol catabolism in fungi proceeds as illustrated in Figure 3.1. This proposed pathway reflects the fungal situation, with genes assigned for the hydrolysis of 4-hydrobenzoate based on characterized genes/enzymes in *Aspergillus niger* (Lubbers *et al.*, 2019; Sgro *et al.*, 2023). In bacteria, the degradation of 3,4-DHB deviates from that of fungi in the steps between 3-carboxy-*cis,cis*-muconate and β -ketoadipate as follows: 3-carboxy-*cis,cis*-muconate cycloisomerase converts 3-carboxy-*cis,cis*-muconic acid into 4-carboxymuconolactone, 4-carboxymuconolactone decarboxylase converts 4-carboxymuconolactone to β -ketoadipate enol-lactone, and β -ketoadipate enol-lactonase converts β -ketoadipate enol-lactone to β -ketoadipate (Ornston & Stanier, 1966).

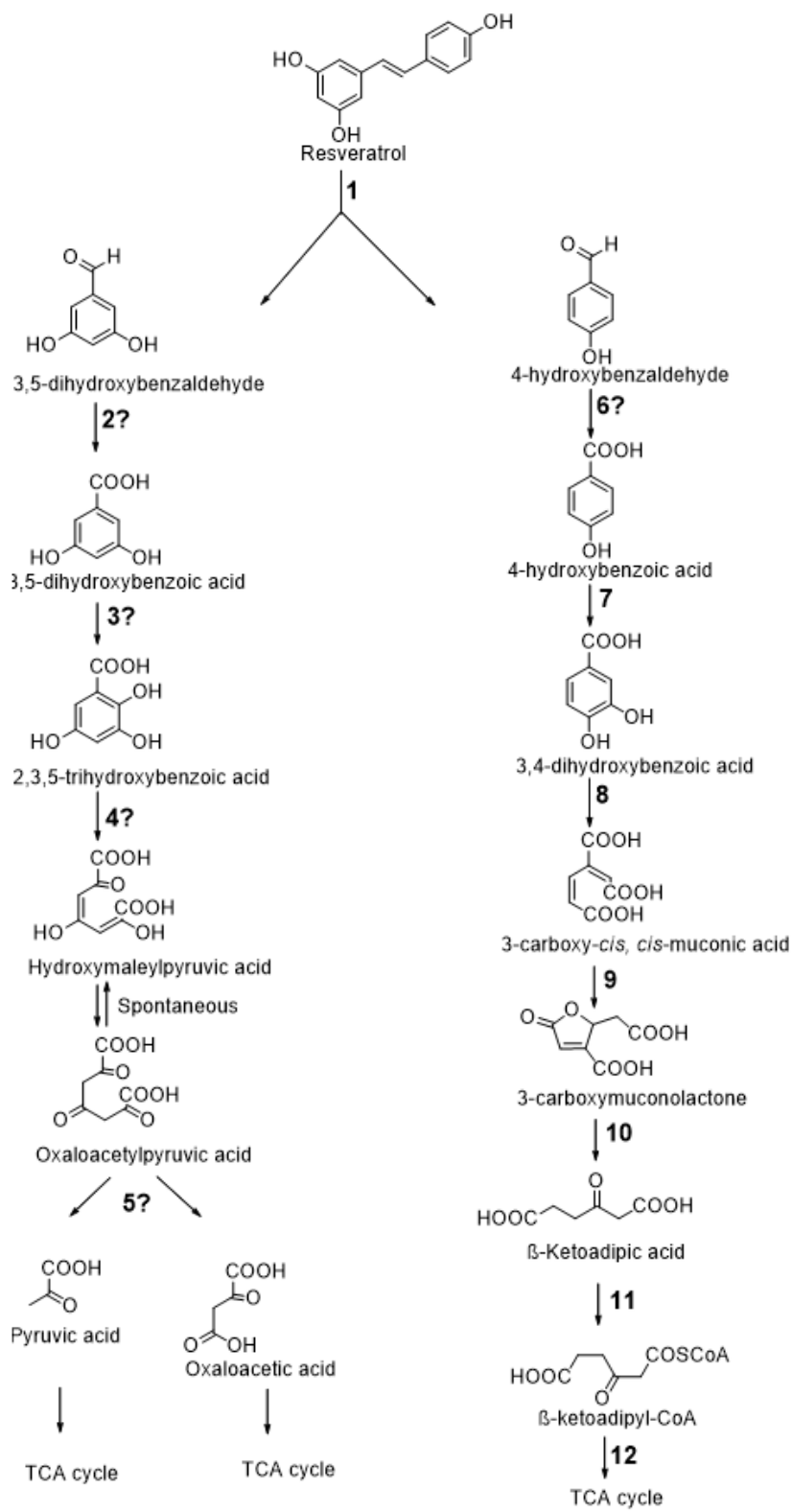


Figure 3.1 Proposed pathway for resveratrol degradation in fungi. Resveratrol has been shown to undergo oxidative cleavage to yield 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde. Proposed are the steps involved in the conversion of the two hydroxybenzaldehydes to TCA cycle intermediates. The numbers indicate the enzyme for each step and are as follows; **1**, resveratrol cleavage oxygenase; **2**, 3,5-dihydroxybenzaldehyde dehydrogenase; **3**, 3,5-dihydrobenzoate 2-monooxygenase; **4**, 2,3,5-trihydroxybenzoate 1,2-dioxygenase; **5**, oxaloacetylpyruvate hydrolase; **6**, 4-hydroxybenzaldehyde dehydrogenase; **7**, *p*-hydroxybenzoate-*m*-hydroxylase; **8**, protocatechuate 3,4-dioxygenase; **9**, 3-carboxy-*cis*, *cis*-muconate cyclase; **10**, 3-carboxymuconolactone hydrolase/decarboxylase; **11**, β -ketoadipate:succinyl-CoA transferase, and **12**, β -ketoadipyl-CoA thiolase. The question marks (?) denote steps where the specific enzymes have not yet been identified.

To date, only the first step of resveratrol degradation, mediated by resveratrol cleavage oxygenase, has been characterized in bacteria and fungi (Kurt *et al.*, 2018; Wang *et al.*, 2019). Resveratrol cleavage oxygenase belongs to a class of enzymes known as lignostilbene- α,β -dioxygenases, also referred to as stilbene cleavage oxygenases. These iron-dependent enzymes are part of a larger family of enzymes called carotenoid cleavage oxygenases (Walter & Strack, 2011). In fungi, the genes encoding carotenoid cleavage oxygenases have been identified from *Aspergillus fumigatus*, *Fusarium fujikuroi*, *Neurospora crassa* and *Ustilago maydis* (Prado-Cabrero *et al.*, 2007; Brefort *et al.*, 2011; Diaz-Sanchez *et al.*, 2013). In the filamentous fungus *Aspergillus niger*, the genes and enzymes involved in the conversion of 4-hydroxybenzoate to 3,4-DHB, *phhA* (NRRL3_08551) (Lubbers *et al.*, 2019), as well as the catabolic pathway of 3,4-DHB (Sgro *et al.*, 2023) have been characterized. In the proposed pathway for resveratrol catabolism (Fig. 3.1), the genes involved in the conversion of benzaldehydes and the subsequent steps of 3,5-DHB degradation have not been identified.

In fungi, pathway genes of metabolites can be organized in gene clusters, which consist of co-localized sets of genes encoding enzymes, regulators and transporters functioning in the same metabolic processes. Genomic and phylogenetic analyses revealed that most fungal stilbene cleavage oxygenases are encoded by members of gene clusters involved in metabolite metabolism (Gluck-Thaler *et al.*, 2018).

In Chapter 2, we previously identified two gene clusters in *Aspergillus niger*, *NRRL3_05233-NRRL3_05237* and *NRRL3_10244-NRRL3_10250*, that were upregulated in the presence of gentisate. We showed that the cluster *NRRL3_05233-NRRL3_05237* encodes genes involved in the gentisate catabolic pathway (Manuscript in preparation).

In this study, we provide evidence from mutational studies and metabolites accumulating in pathway mutants to show that the cluster *NRRL3_10244-NRRL3_10250* encodes genes for resveratrol cleavage oxygenase and enzymes involved in the degradation of one of its two products, 3,5-dihydroxybenzaldehyde. In addition, we showed that the previously characterized vanillin dehydrogenase *VdhA* (Lubbers *et al.*, 2024) catalyzes both 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde to 3,5-DHB and 4-hydroxybenzoate, respectively. We further confirmed that the protocatechuate catabolic pathway is involved in the catabolism of 4-hydroxybenzaldehyde. These results allow us to assign the complete set of genes involved in the proposed catabolic pathway of resveratrol in *A. niger*.

3.3 Results and Discussion

3.3.1 Predicted function of the *NRRL3_10244–NRRL3_10250* gene cluster of *A. niger*

In Chapter 2, we presented two paralogous gene clusters in *A. niger*, one of which encodes genes involved in gentisate catabolism. Genes in both clusters are upregulated in the presence of gentisate. Based on the upregulation of transcripts, we initially defined the second cluster as encompassing seven genes, extending from *NRRL3_10244* to *NRRL3_10250*. To predict the function of the genes in the cluster, we analyzed their protein domain structures using the Pfam protein family database (Mistry *et al.*, 2021) as well as using BLASTP to search the UniProtKB/Swiss-Prot database. The predicted functions of the proteins encoded by the clustered genes are: *NRRL3_10244*, gentisate-1,2-dioxygenase; *NRRL3_10245*, FAD-dependent monooxygenase; *NRRL3_10246*, fungal-specific transcription factor; *NRRL3_10247*, carotenoid cleavage oxygenase; *NRRL3_10248*, fumarylacetoacetase; *NRRL3_10249*, major facilitator superfamily transporter; and *NRRL3_10250*, lysophospholipase. The predicted functions of the clustered genes, except for the *NRRL3_10250* lysophospholipase, suggest that they may be involved in the proposed catabolism of resveratrol.

Gene clusters in fungi evolve rapidly. Genes involved in the same metabolic pathway can be co-localized in some species, while they are unlinked in related species. For example, the genes responsible for pigment production are clustered in *A. fumigatus*, while in *A. niger*, these genes are scattered throughout the genome (Chiang *et al.*, 2011). Likewise, the gene clusters involved in alkylcitrates biosynthesis in *A. oryzae* and quinate catabolism in *A. nidulans* are fragmented in *A. niger* (Palys *et al.*, 2020; Sgro *et al.*, 2025). To define the boundary of the gene cluster more precisely and to uncover off-cluster pathway genes, we compared the *NRRL3_10244* to *NRRL3_10250* cluster of *A. niger* with orthologous clusters in Aspergilli. Few *Aspergillus* species contain such a gene cluster, and they are scattered throughout the phylogenetic tree. Figure 3.2 shows the synteny map of select orthologous clusters. The core cluster encompasses *NRRL3_10244* to *NRRL3_10249*, as the orthologues of *NRRL3_10250*, encoding a predicted lysophospholipase, are not in synteny with the orthologous clusters. The gene *NRRL3_10249* is predicted to encode a major facilitator superfamily transporter, suggesting that it may play a role in the transport of the metabolite that is under the regulation of the cluster. This leaves *NRRL3_10244-NRRL3_10248* as the portion of the cluster that encodes enzymes and a regulator. The three orthologous clusters shown in Figure 3.2 include a gene predicted to encode aldehyde dehydrogenase, which is not present in *A. niger* cluster. In the proposed resveratrol catabolic pathway (Fig. 3.1), two benzaldehyde dehydrogenases are predicted.

In Chapter 2, we constructed a cluster knockout strain with genes *NRRL3_10244-NRRL3_10250* deleted. This deletion strain does not affect the growth of *A. niger* on gentisate (Chapter 2). Figure 3.3 shows that $\Delta NRRL3_10244-NRRL3_10250$ was unable to grow on resveratrol as the sole carbon source, confirming that this cluster contains genes involved in the catabolism of resveratrol.

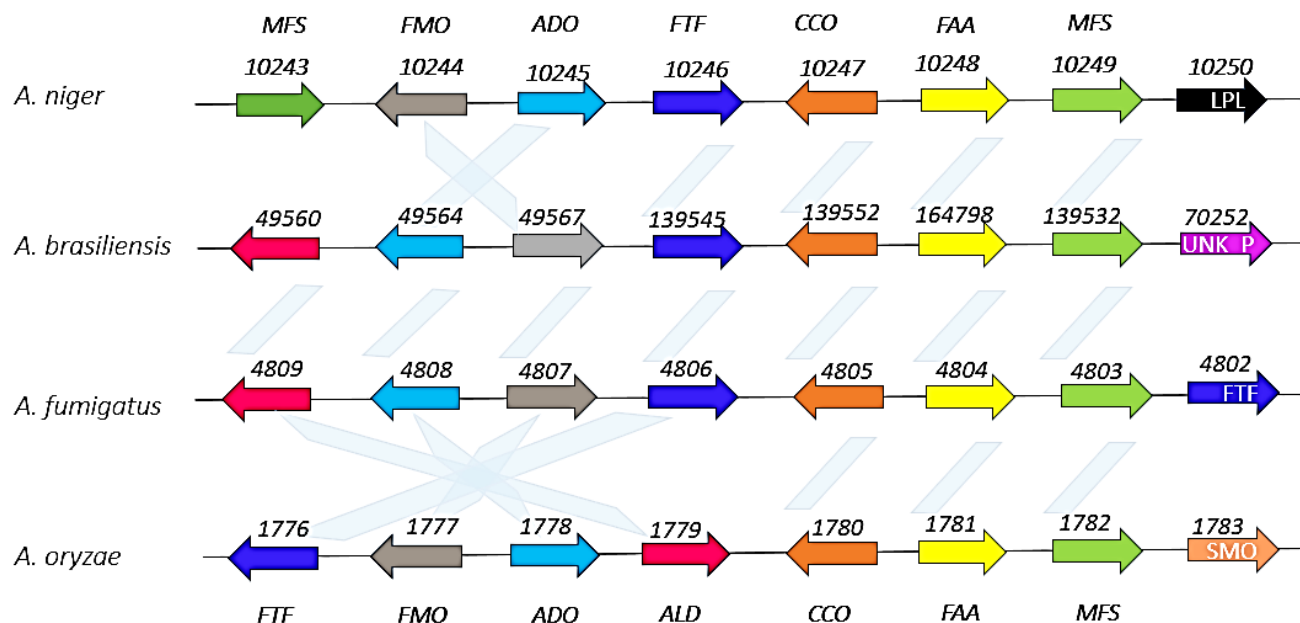


Figure 3.2 Gene cluster organization for predicted resveratrol catabolism in *Aspergillus* species. Predicted gene functions are assigned based on protein domain architecture and orthology analysis. Shaded connectors indicate homologous genes across species. The identified enzymes include major facilitator superfamily transporter (**MFS**), FAD-dependent monooxygenase (**FMO**), aromatic-ring cleavage dioxygenase (**ADO**), fungal-specific transcription factor (**FTF**), carotenoid cleavage oxygenase (**CCO**), fumarylacetoacetase family protein (**FAA**), lysophospholipase (**LPL**), C-4 sterol methyl oxidase (**SMO**), and a protein of unknown function (**UNK_P**). A conserved aldehyde dehydrogenase (**ALD**) is co-localized within the cluster in three species but is absent from *A. niger*. The numbers represent gene IDs of *A. niger* NRRL3 and *A. brasiliensis* v1.0 obtained from MycoCosm of the Joint Genome Institute, *A. fumigatus* Af293 and *A. oryzae* RIB40 from the *Aspergillus* Genome database (Arnaud et al., 2012).

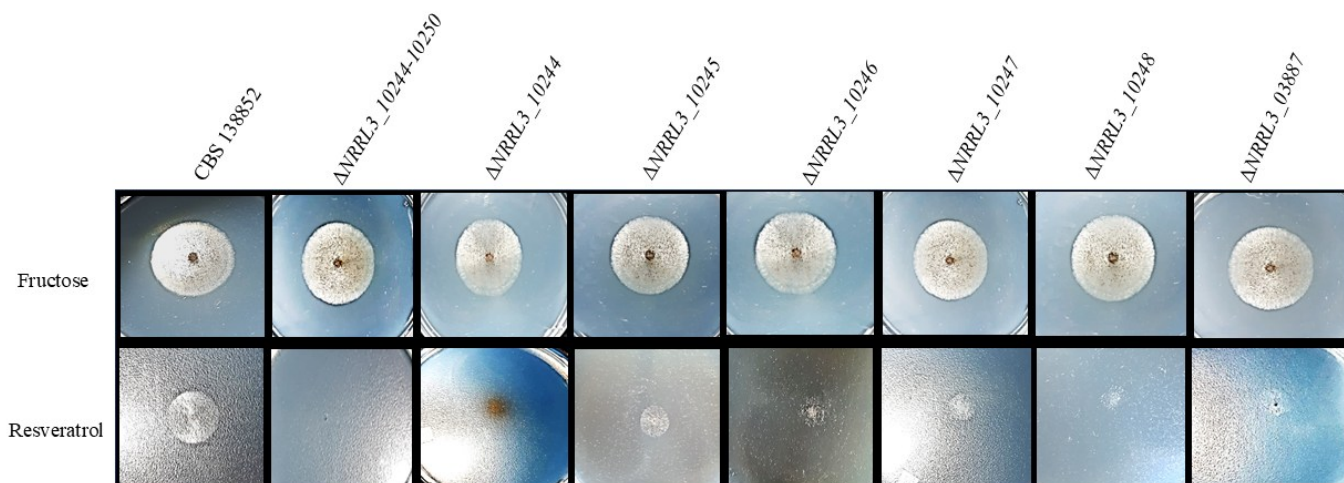


Figure 3.3 Growth phenotype of *A. niger* parent strain and resveratrol pathway mutants. Parent and mutant strains were grown on agar plates with fructose or resveratrol as a carbon source.

3.3.2 *NRRL3_10247* encodes resveratrol cleavage dioxygenase *RcoA*

The first step of the resveratrol catabolic pathway in bacteria and fungi has been characterized, and it involves the oxidative cleavage of the resveratrol double bonds, yielding two hydroxybenzaldehyde products (Figure 3.1). The *cao-1* gene of *N. crassa* has been shown to encode resveratrol cleavage oxygenase (Diaz-Sanchez *et al.*, 2013). We used the Cao-1 sequence of *N. crassa* as a query for a BLASTP search of the *A. niger* NRRL3 genome and identified two close orthologues: NRRL3_10247 and NRRL3_00108.1, with sequence identity of 62% and 58%, respectively. The gene *NRRL3_00108.1* is a member of a predicted biosynthetic gene cluster involved in secondary metabolite production (Semper *et al.*, 2025). This leaves *NRRL3_10247* as the likely candidate for encoding a resveratrol cleavage dioxygenase. Deletion of *NRRL3_10247* resulted in reduced growth on resveratrol (Fig. 3.3).

We used liquid chromatography-mass spectrometry (LC-MS) to analyze metabolites accumulated in the extracellular medium during cultivation of pathway mutants on resveratrol. Resveratrol utilization in the Δ NRRL3_10247 mutant was reduced, leading to substantially higher extracellular resveratrol levels compared to the parent strain, which efficiently consumed the compound within 6 hours (Figure 3.4). This result aligns with the expected impact on a mutant lacking resveratrol cleavage dioxygenase activity. Based on sequence similarity to the

characterized resveratrol cleavage oxygenase of *N. crassa*, the reduced growth of $\Delta NRRL3_10247$ on resveratrol, and the reduced rate of consumption of resveratrol of $\Delta NRRL3_10247$, we designate *NRRL3_10247* as *RcoA*, encoding resveratrol-cleaving dioxygenase.

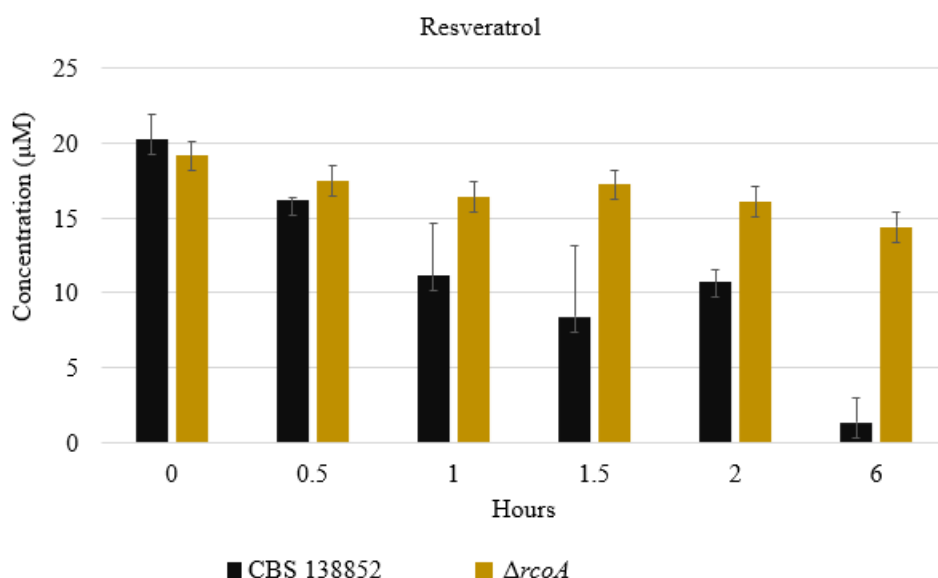


Figure 3.4 Consumption of resveratrol in the $\Delta rcoA$ ($\Delta NRRL3_10247$) strain, and *A. niger* parent strain; Both strains were cultivated on minimal medium with resveratrol as the sole source of carbon.

Since resveratrol cleavage oxygenase acts on the first step of resveratrol catabolism, before its two products are catabolized by separate pathways, we expect $\Delta rcoA$ to abolish growth on resveratrol. The growth phenotype and the reduced substrate consumption of $\Delta rcoA$ would suggest that resveratrol is utilized at a reduced rate in $\Delta rcoA$. These results imply that another gene or pathway may compensate, albeit inefficiently, for the loss of *rcoA* in resveratrol utilization in *A. niger*.

3.3.3 *NRRL3_03887* (*vdhA*) encodes a versatile benzaldehyde dehydrogenase

Aldehyde dehydrogenases oxidize aldehydes to their corresponding carboxylic acids, typically using cofactor NAD^+ or $NADP^+$ (Shortall *et al.*, 2021; Jeong *et al.*, 2023). They play essential roles in aromatic compound metabolism, particularly in the degradation of lignin-derived

intermediates such as vanillin, syringaldehyde, and benzaldehyde (Kamimura *et al.*, 2017; Lubbers & de Vries, 2021). We propose (Figure 3.1) that two hydroxybenzaldehyde dehydrogenases participate in the catabolism of resveratrol, one converting 3,5-dihydroxybenzaldehyde to 3,5-dihydrobenzoate and the other from 4-hydroxybenzaldehyde to 4-hydroxybenzoate (Figure 3.1). While the *NRRL3_10244-NRRL3_10250* gene cluster does not contain a predicted aldehyde dehydrogenase gene, its orthologous clusters in *A. brasiliensis*, *A. fumigatus*, and *A. oryzae* include an aldehyde dehydrogenase gene despite rearrangement of the clustered genes between these species (Figure 3.2). We used the in-cluster aldehyde dehydrogenase from *A. brasiliensis*, *A. fumigatus*, and *A. oryzae* to perform BLASTP searches against the *A. niger* NRRL3 genome. In each search, the top hit was a previously characterized vanillin dehydrogenase VdhA (NRRL3_03887) (Lubbers *et al.*, 2024), with sequence identity of 51%-55%. The *A. niger* VdhA has a broad substrate spectrum with strong activity on vanillin, benzaldehyde, *p*-hydroxybenzaldehyde and protocatechuic aldehyde (Lubbers *et al.*, 2024).

To determine its role in resveratrol catabolism, we deleted the *vdhA* gene and cultivated the deletion strains on resveratrol and the two resveratrol-derived metabolites 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde. Figures 3.3 and 3.5 show that the growth of $\Delta vdhA$ was severely impaired on resveratrol and 3,5-dihydroxybenzaldehyde but less affected on 4-hydroxybenzaldehyde. Reduced, instead of the abolition of growth of $\Delta vdhA$ on 4-hydroxybenzaldehyde had been observed previously (Lubbers & de Vries, 2021), suggesting the possible involvement of an alternative enzyme or pathway with partial activity on 4-hydroxybenzaldehyde. The severe growth defect of $\Delta vdhA$ on 3,5-dihydroxybenzaldehyde suggests that *vdhA* is essential for the *in vivo* conversion of 3,5-dihydroxybenzaldehyde to 3,5-DHB. The unimpaired growth of $\Delta vdhA$ on 4-hydroxybenzoic acid was expected because 4-hydroxybenzoic acid is a product of *VdhA*. However, we have no explanation for the reduced growth of $\Delta vdhA$ on 3,5-DHB (Figure 3.5) because VdhA is not predicted to be involved in the catabolism of 3,5-DHB.

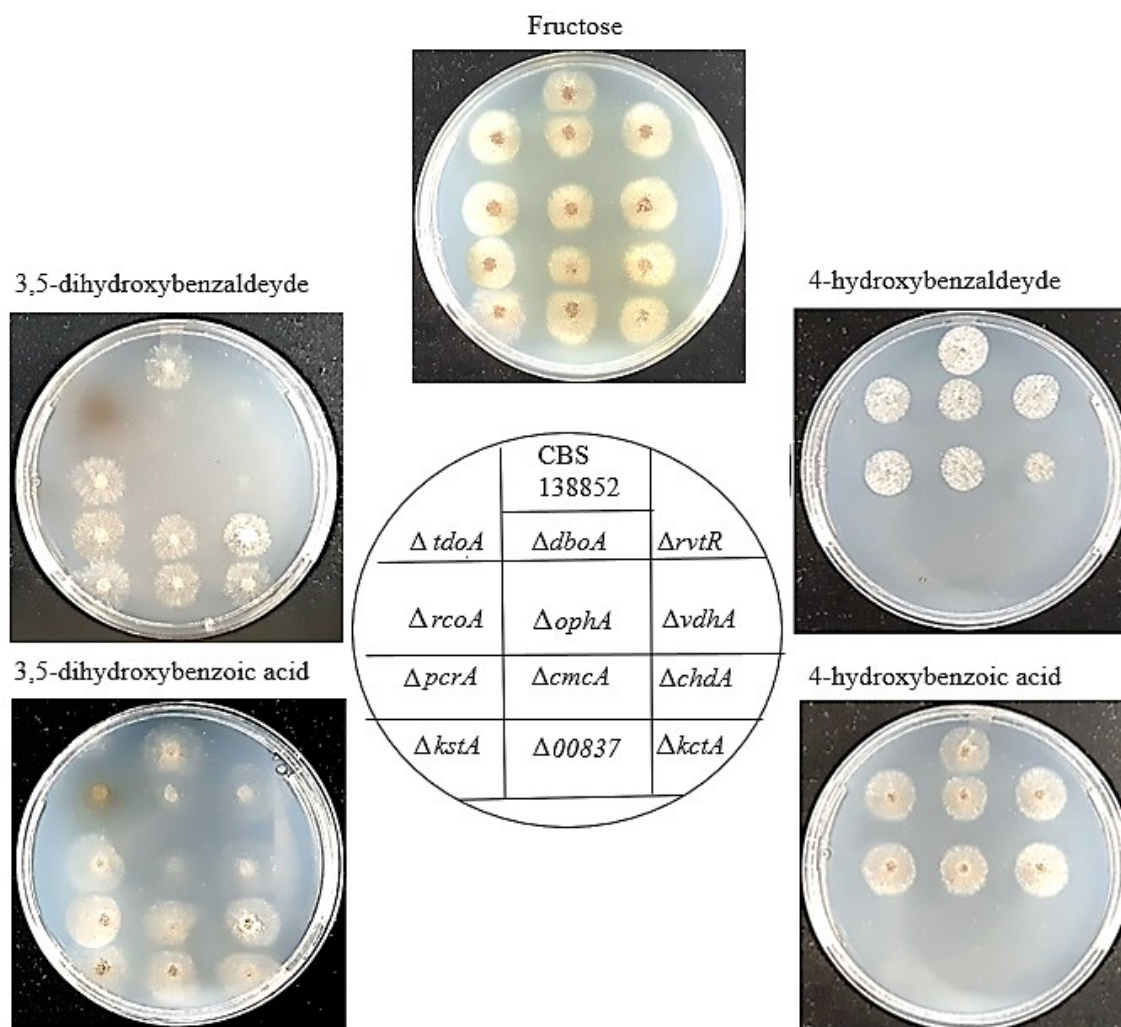
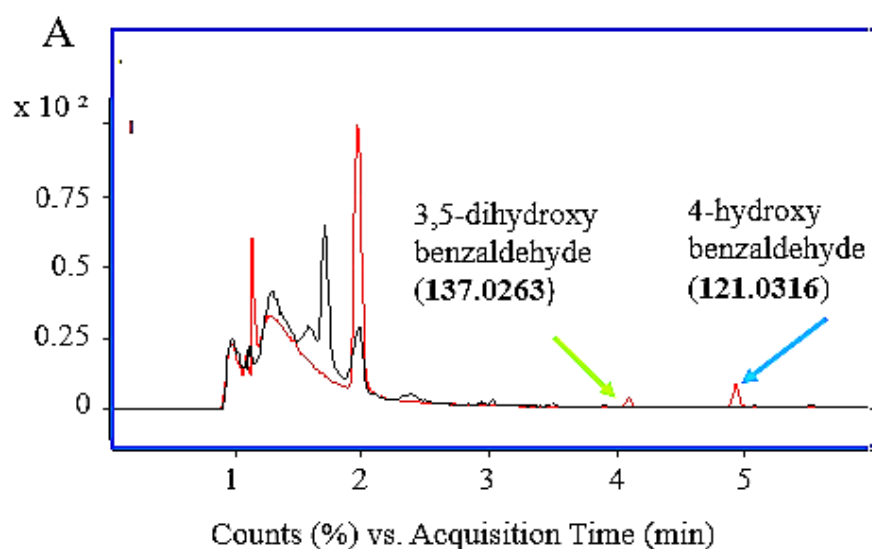


Figure 3.5 Growth phenotype of *A. niger* mutants on hydroxybenzene compounds.

Deletion mutants of predicted resveratrol pathway genes $\Delta tdoA$ (NRRL3_10244), $\Delta dboA$ (NRRL3_10245), $\Delta rvtR$ (NRRL3_10246), $\Delta rcoA$ (NRRL3_10247), $\Delta ophA$ (NRRL3_10248), $\Delta vd hA$ (NRRL3_03887) and genes from the protocatechuate branch of the beta-ketoadipate pathway ($\Delta pcrA$, $\Delta cmcA$, $\Delta chdA$, $\Delta kstA$, $\Delta 00837$ and $\Delta kctA$) (Sgro et al., 2023) were cultivated on minimal medium containing the indicated aromatic compounds as sole carbon sources. The resveratrol pathway branches into two routes: one through 4-hydroxybenzoate (via 3,4-dihydroxybenzoate) and another through 3,5-dihydroxybenzoate. Fructose served as a control for normal growth.

To further support the physiological role of *vdhA* in the conversion of both 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde, we quantified the accumulation of resveratrol cleavage products in the $\Delta vdhA$ mutant. The MS profile (Figure 3.6A-C) revealed distinct peaks corresponding to metabolites that accumulated in the $\Delta vdhA$ mutant on resveratrol but were absent in the parent strain. Two signals were detected with $m/z = 121.0316$ ($[M-H]^-$) and $m/z = 137.0263$ ($[M-H]^-$), matching the expected negative ionization m/z values for 4-hydroxybenzaldehyde (121.0295) and 3,5-dihydroxybenzaldehyde (137.0244), respectively.



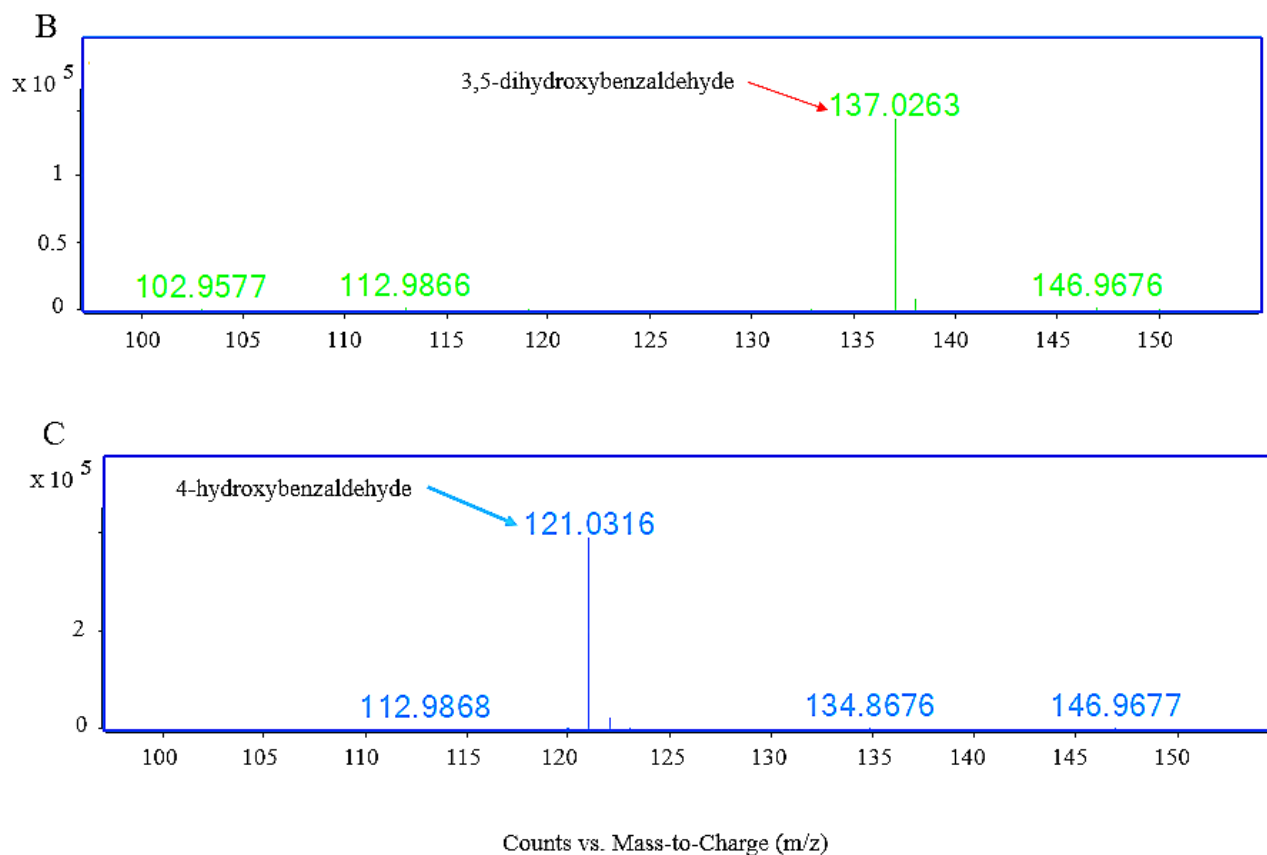
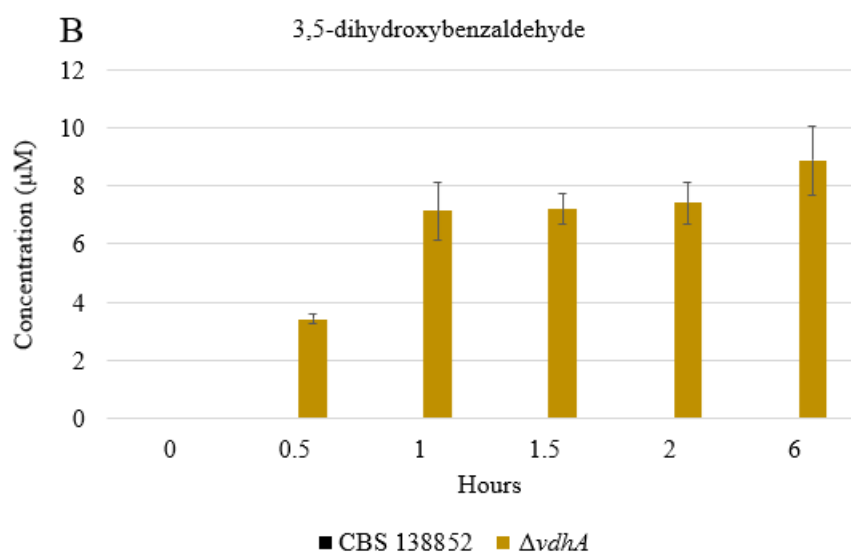
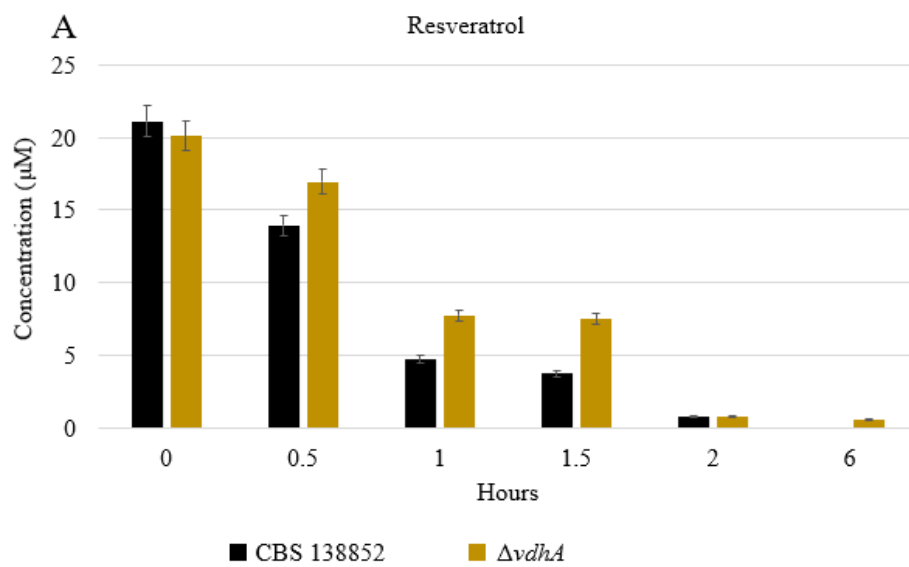


Figure 3.6 LC–MS profiles of $\Delta vd hA$ and parent strain grown on resveratrol.

Panel A shows the total ion chromatogram (TIC) of extracellular extracts from the mutant strain $\Delta vd hA$ (red) and the *A. niger* parent strain (black). Peaks at retention times corresponding to 3,5-dihydroxybenzaldehyde (m/z 137.0263) and 4-hydroxybenzaldehyde (m/z 127.0316) were detected only in the mutant extract, $\Delta vd hA$. Panels B and C show the MS spectra of the two compounds with mass-to-charge (m/z) values expected for 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde.

Metabolite analysis of the extracellular medium further showed that both the mutant and parental strains consumed resveratrol at comparable rates (Figure 3.7A). However, only the $\Delta vd hA$ mutant accumulated detectable amounts of 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde, where the concentrations of these compounds increased over time following transfer to medium containing resveratrol.



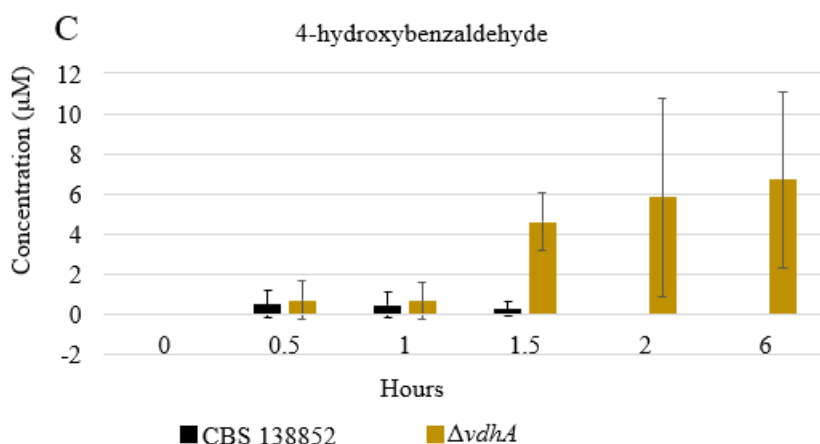
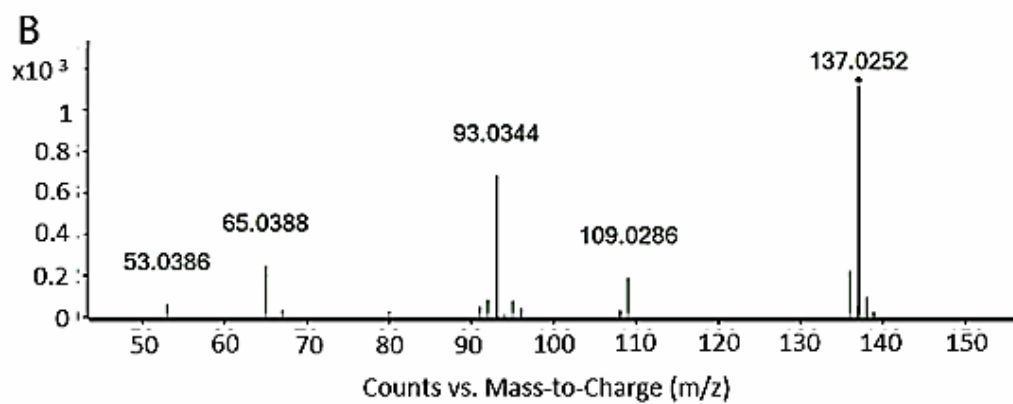
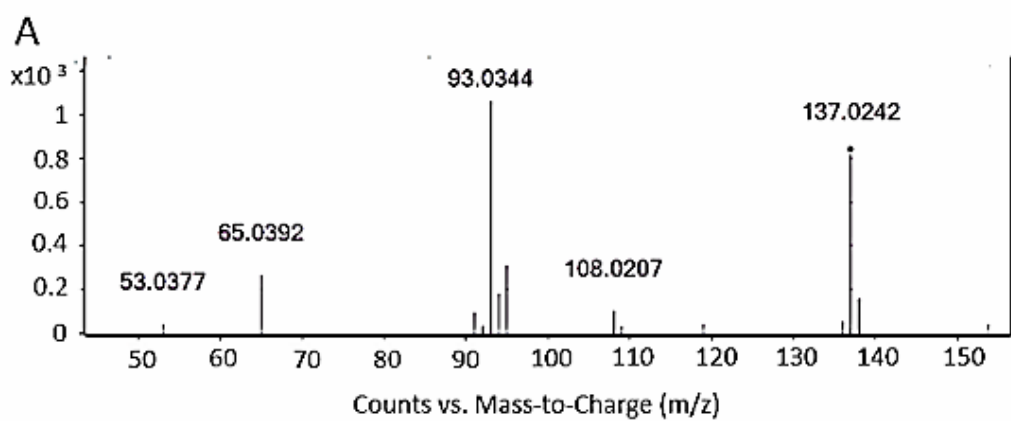


Figure 3.7 Accumulation of 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde by $\Delta vd hA$ ($\Delta NRRL3_03887$). (A) Consumption of resveratrol in *A. niger* parent strain and mutant $\Delta vd hA$ strain cultivated in minimal medium with resveratrol as the sole source of carbon; (B) accumulation of 3,5-dihydroxybenzaldehyde in parent strain and $\Delta vd hA$; (C) accumulation of 4-hydroxybenzaldehyde in parent strain and $\Delta vd hA$

We used MS/MS to further confirm that the metabolites accumulated in the $\Delta vd hA$ strain are 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde by comparing their fragmentation patterns with those of commercially available compounds. The fragmentation spectra of the products accumulated in $\Delta vd hA$ were similar to the reference 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde compounds (Figure 3.8A-D), thereby supporting the conclusion that *vdhA* is involved in the *in vivo* conversion of both 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde to their respective hydroxybenzoates.



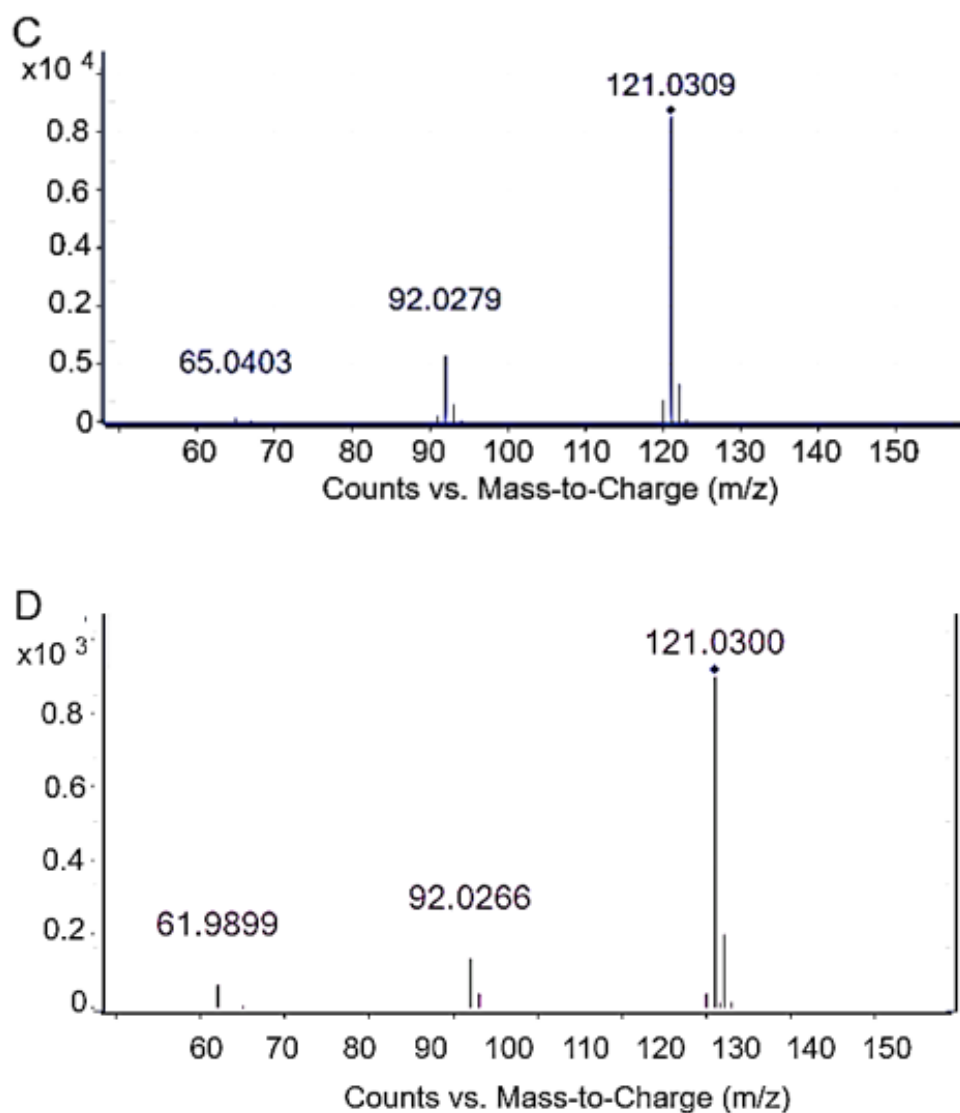


Figure 3.8 Comparison of MS/MS fragmentation spectra of reference hydroxybenzaldehydes and compounds accumulated in $\Delta vdhA$. (A) Reference compound 3,5-dihydroxybenzaldehyde (3,5-DHBZA) compared to (B) 3,5-DHBZA accumulated in the $\Delta vdhA$ strain, and (C) reference compound 4-hydroxybenzaldehyde (4-HBZA) compared to (D) 4-HBZA accumulated in the $\Delta vdhA$ strain.

The vanillin dehydrogenase VdhA of *A. niger* is active on a broad range of aromatic aldehydes with a preference for hydroxybenzaldehydes, including vanillin, 4-hydroxybenzaldehyde and protocatechuic aldehyde. Transcriptome analysis showed that the *vdhA*

gene is upregulated under exposure to different aromatic compounds. Deletion of *vdhA* abolished growth on vanillin and reduced growth on 4-hydroxybenzaldehyde and protocatechuic aldehyde (Lubbers *et al.*, 2021; Lubbers *et al.*, 2024). In addition to confirming the role of *vdhA* in the conversion of 4-hydroxybenzaldehyde, this study demonstrated that *vdhA* is essential for the *in vivo* conversion of 3,5-dihydroxybenzaldehyde in *A. niger*. Taken together, these results suggest that VdhA is a versatile hydroxybenzaldehyde dehydrogenase that is required for multiple catabolic pathways, including vanillin and resveratrol.

The orthologue of *vdhA* in *A. brasiliensis*, *A. fumigatus* and *A. oryzae* is located at the resveratrol catabolic gene cluster (Figure 3.2). This would imply that in these species, the *vdhA* orthologue is under the control of the resveratrol regulator. In *A. niger*, *vdhA* is not linked to the resveratrol catabolic gene cluster. Additionally, *vdhA* is upregulated when cultured under different aromatics (Lubbers *et al.*, 2021), suggesting that it is under the control of multiple regulators.

3.3.4 $\Delta NRRL3_10245$ mutant accumulates 3,5-dihydroxybenzoate

The products of VdhA in the resveratrol catabolic pathway are expected to be 3,5-DHB and 4-hydroxybenzoic acid (4-HB). To elucidate the function of the enzyme encoded by *NRRL3_10245* and its involvement in the resveratrol catabolic pathway, we deleted *NRRL3_10245*. Figure 3.3 shows the growth phenotype of the $\Delta NRRL3_10245$ mutant on resveratrol and resveratrol-derived metabolites (Figure 3.5). Strain $\Delta NRRL3_10245$ displayed impaired growth on 3,5-dihydroxybenzaldehyde but no growth defect on 4-hydroxybenzaldehyde, suggesting that *NRRL3_10245* is involved in the 3,5-dihydroxybenzaldehyde part of the resveratrol catabolic pathway. The defective growth phenotype of $\Delta NRRL3_10245$ when grown on 3,5-DHB suggests that *NRRL3_10245* encodes the enzyme required to catabolize 3,5-DHB or one of its degradative products.

To further determine the function of *NRRL3_10245*, we analyzed the LC-MS profile, which shows compounds accumulated in the $\Delta NRRL3_10245$ mutant but absent in the parent strain. One accumulated compound in $\Delta NRRL3_10245$ has an m/z value of 153.0198 ([M-H]⁻), matching the theoretical m/z of 153.0190 for 3,5-DHB in the negative mode. This compound was not detected in the parent strain (Figure 3.9A-B).

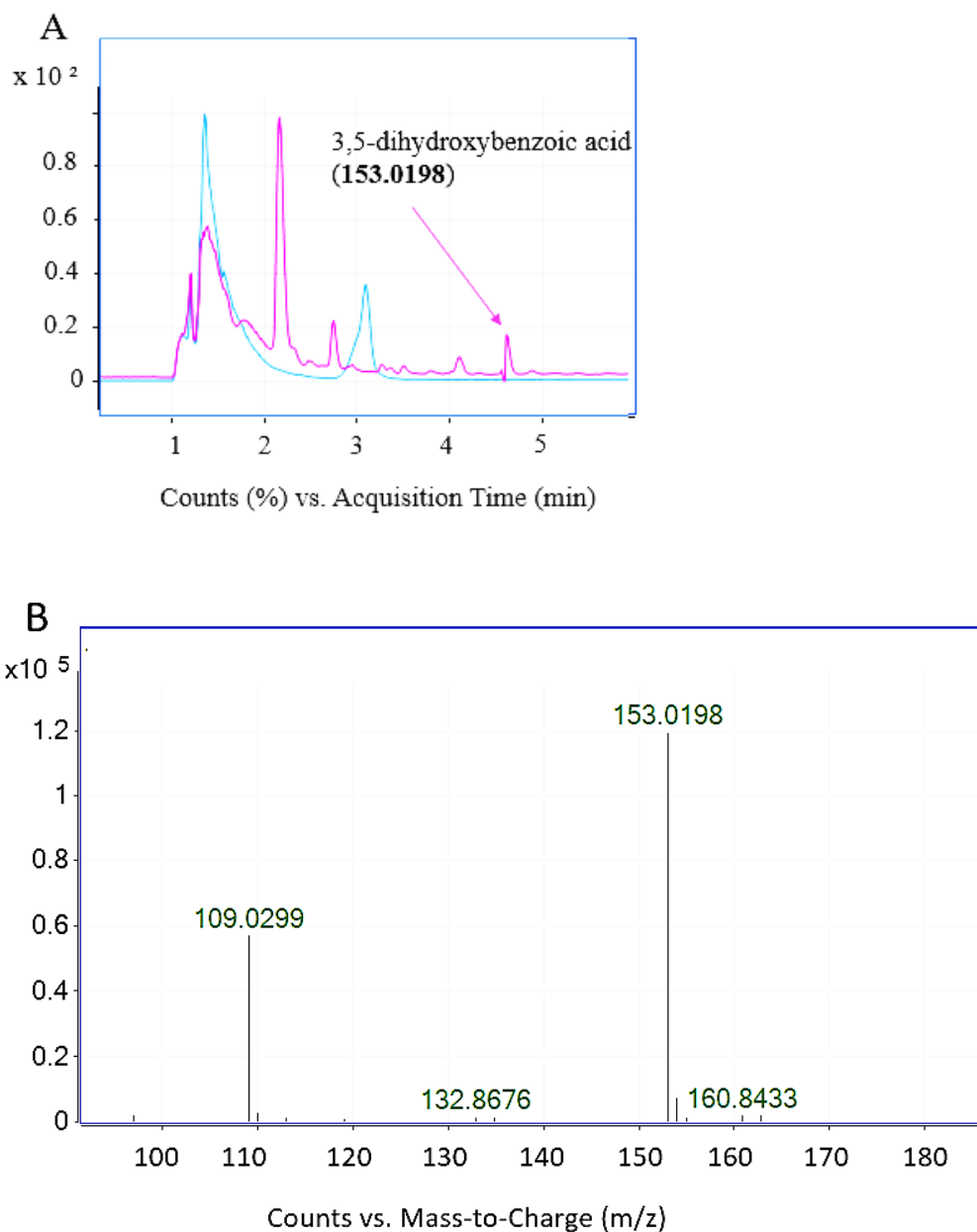


Figure 3.9 LC–MS profiles of Δ dboA (Δ NRRL3_10245) and parent strain grown on resveratrol. Panel A shows the total ion chromatogram (TIC) of extracellular extracts from the mutant strain, Δ dboA (magenta) and the parent strain (blue). A peak at retention time corresponding to 3,5-dihydroxybenzoic acid (m/z 153.0198) was detected only in the Δ dboA mutant extract. Panel B, MS spectra showing the mass-to-charge (m/z) value expected for 3,5-dihydroxybenzoic acid.

Analysis of metabolites in the extracellular medium shows that while the consumption rate was faster in the mutant strain, the intermediate was detected only in the mutant strain, as shown in Figure 3.10

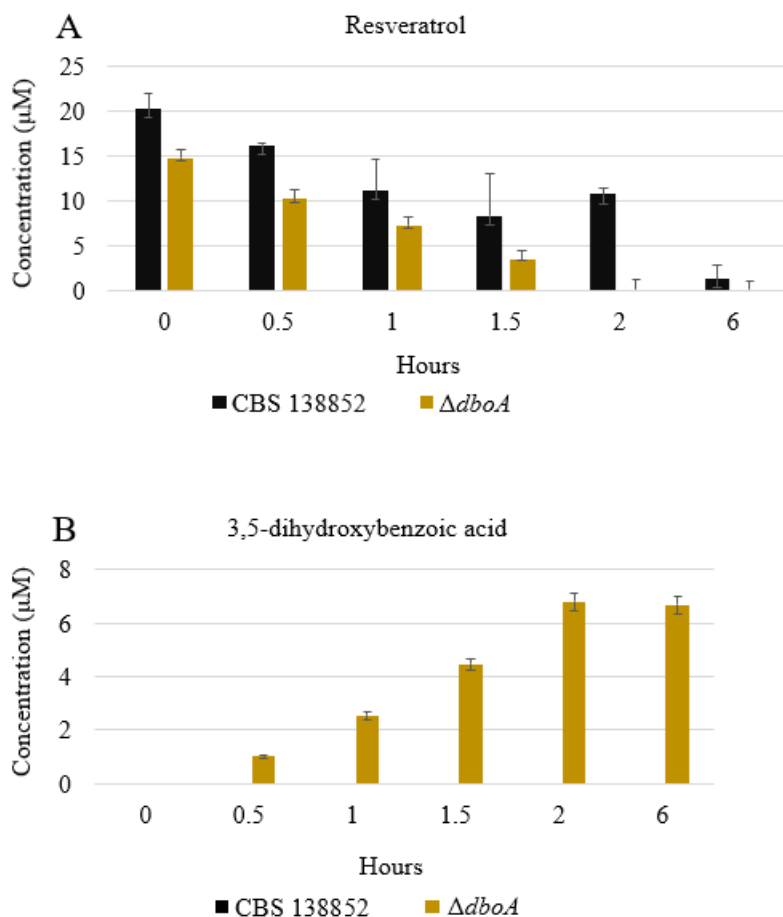


Figure 3.10 Accumulation of 3,5-DHB by $\Delta dbaA$ ($\Delta NRRL3_10245$). LC-MS analysis of (A) utilization of resveratrol in *A. niger* parent strain and mutant strain ($\Delta dbaA$), (B) detection of accumulated product 3,5-DHB in parent strain and $\Delta dbaA$.

Furthermore, a comparison to the reference 3,5-DHB showed that the MS/MS fragmentation pattern of 3,5-DHB was similar to the product accumulated in $\Delta NRRL3_10245$ (Figure 3.11). Since the VdhA product of 3,5-dihydroxybenzaldehyde is predicted to be 3,5-DHB, the accumulation of 3,5-DHB in $\Delta NRRL3_10245$ suggests that NRRL3_10245 acts at a step immediately following VdhA. Amino acid sequence analysis predicted that NRRL3_10245 is a

FAD-dependent monooxygenase. This would imply that NRRL3_10245 adds an oxygen atom to 3,5-DHB and likely converts it to a trihydroxybenzoic acid.

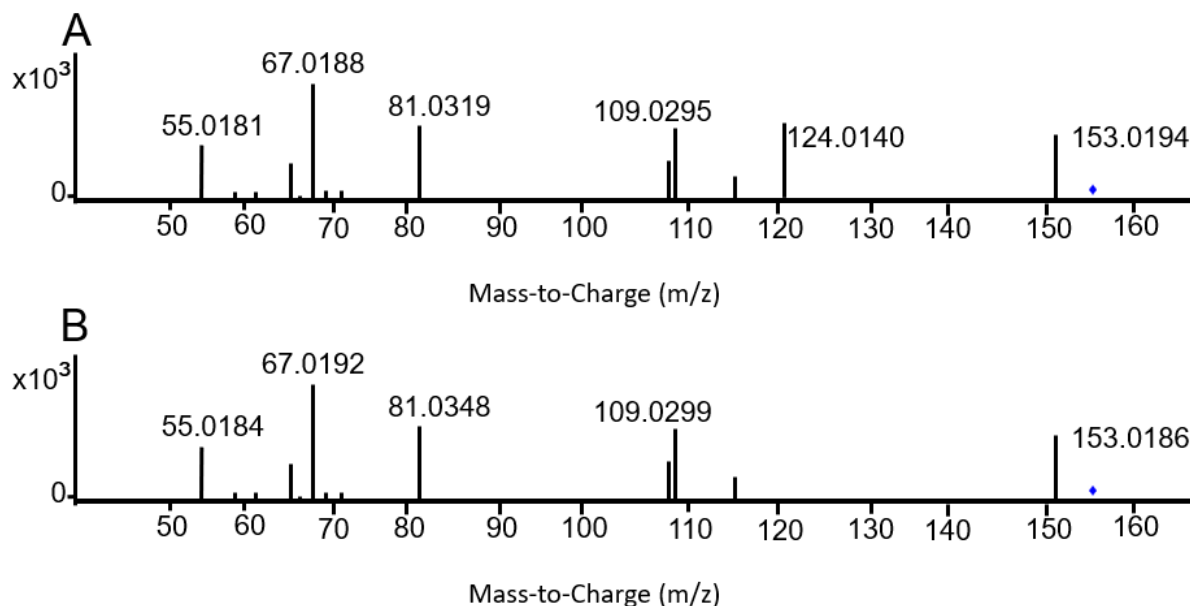
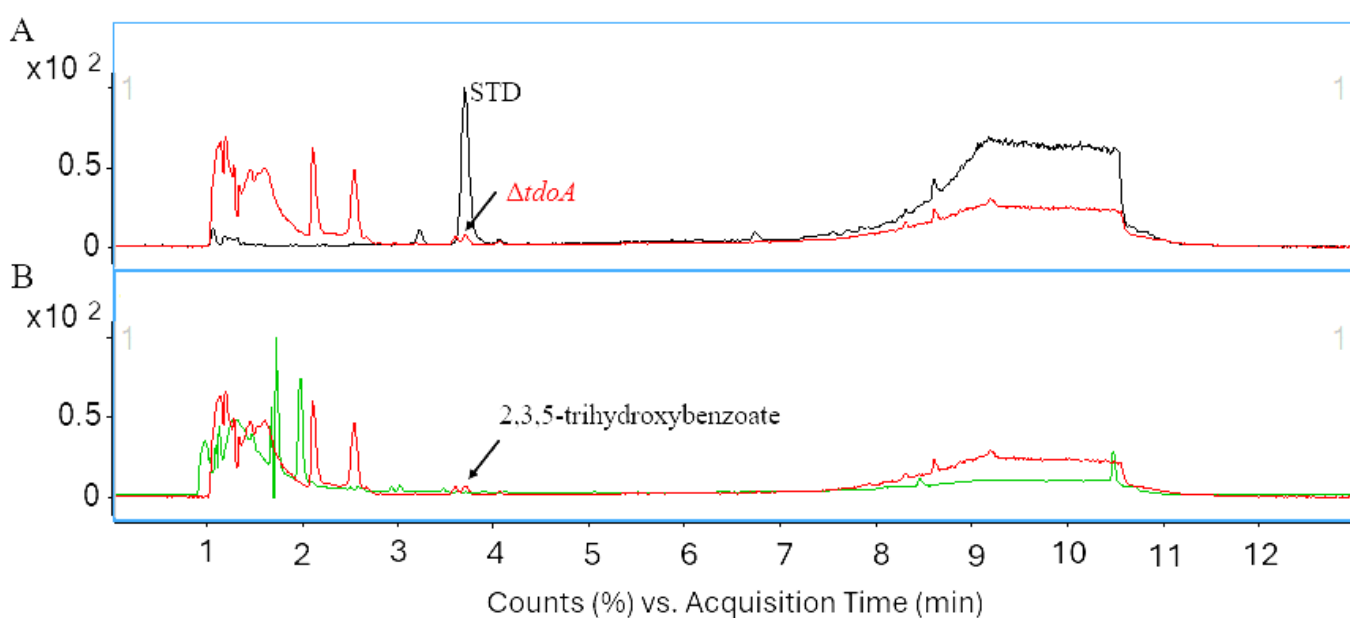


Figure 3.11 MS/MS fragmentation pattern of 3,5-DHB compared to the accumulated metabolite in the Δ dboA (Δ NRRL3_10245) strain. (A) Fragment spectrum of the 3,5-dihydroxybenzoic acid reference compound. (B) Fragment spectrum of the accumulated metabolite in the Δ dboA strain.

3.3.5 Δ NRRL3_10244 accumulates 2,3,5-trihydroxybenzoic acid

NRRL3_10244 is a paralogue of gentisate 1,2-dioxygenase (Chapter 2), suggesting that it is a 1,2-dioxygenase. We deleted NRRL3_10244, and the growth of Δ NRRL3_10244 on resveratrol-derived substrates is shown in Figure 3.3. The mutant Δ NRRL3_10244 exhibited reduced growth on 3,5-dihydroxybenzaldehyde and 3,5-DHB (Figure 3.5), suggesting that it is involved in a step downstream of NRRL3_10245 in the catabolism of resveratrol. The mutant Δ NRRL3_10244 accumulated a brown-coloured compound on resveratrol (Fig. 3.3), 3,5-dihydroxybenzaldehyde and 3,5-DHB (Figure 3.5). Commercially available 2,3,5-trihydroxybenzoic acid (2,3,5-THB) is brown in colour. The accumulation of a brown compound by Δ NRRL3_10244 suggests that the mutant accumulates 2,3,5-THB. Figure 3.12A-D shows a

distinct peak in the $\Delta NRRL3_10244$ that elutes at the same retention time as standard 2,3,5-THB. A metabolite with an observed ion at $m/z = 169.0153$ ($[M-H]^-$), corresponding to the molecular mass of 2,3,5-THB in the negative mode, was detected in the $\Delta NRRL3_10244$ strain but was absent in the parent strain. The mass spectrum of this metabolite closely matched that of the 2,3,5-THB standard. Figure 3.13 shows the accumulation of 2,3,5-THB over time when $\Delta NRRL3_10244$ was cultured on resveratrol.



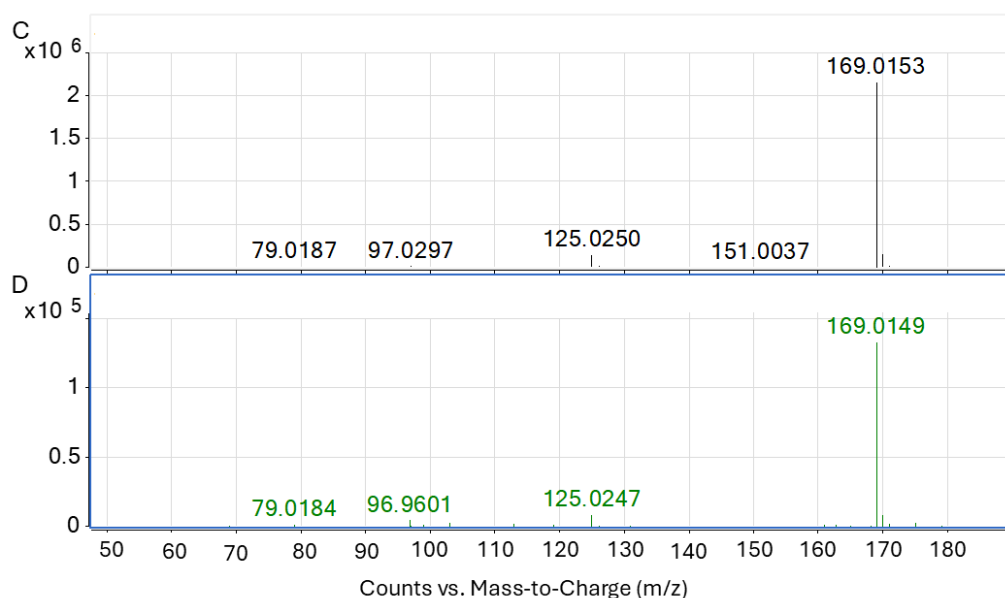
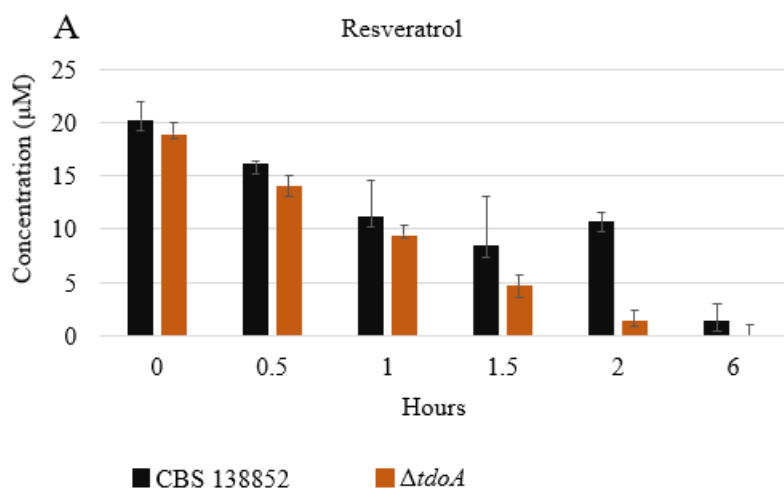


Figure 3.12 LC-MS analysis of pathway intermediate detected in $\Delta tdoA$ ($\Delta NRRL3_10244$) grown on resveratrol. (A) Total ion chromatogram (TIC) of 2,3,5-THB (STD, black peak) and $\Delta tdoA$ (red peak) with similar retention time. (B) TIC of *A. niger* parent strain (green peak) and $\Delta tdoA$ (red peak) at 6 hours of incubation, (C) mass spectrum of standard 2,3,5-THB, and (D) mass spectrum of 2,3,5-THB detected in $\Delta NRRL3_10244$ showing similar spectra



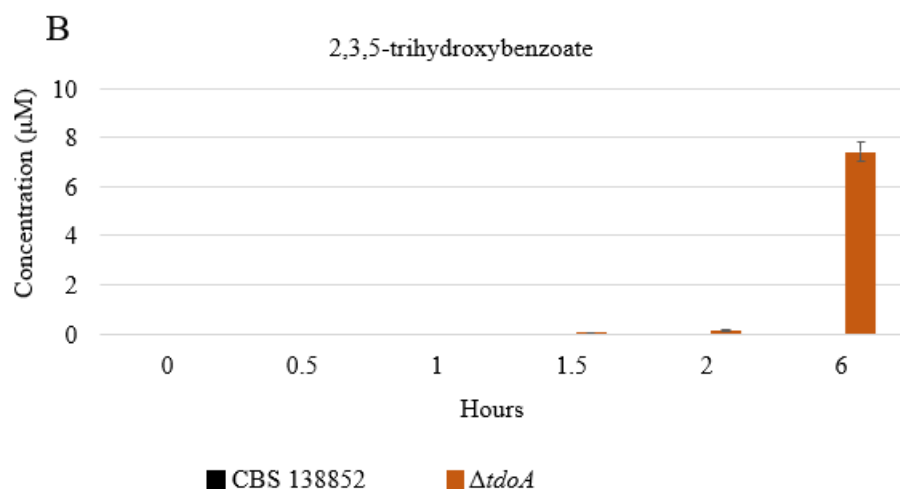


Figure 3.13 Resveratrol consumption and accumulation of 2,35-THB in $\Delta tdoA$ ($\Delta NRRL3_10244$) by LC-MS. (A) utilization of resveratrol in *A. niger* parent strain and $\Delta tdoA$ ($\Delta NRRL3_10244$), (B) detection of accumulated product (2,3,5-trihydroxybenzoate) in the parent strain and $\Delta tdoA$.

In *Rhodococcus jostii* and *B. brevis*, 2,3,5-THB was identified as the first intermediate in the catabolism of 3,5-DHB (Crawford & Perkins, 1978; Montersino & van Berkel, 2012). In the presence of resveratrol, $\Delta NRRL3_10245$ accumulated 3,5-DHB (from 3,5-dihydroxybenzaldehyde through the action of VdhA), and $\Delta NRRL3_10244$ accumulated 2,3,5-THB. Together with the prediction that NRRL3_10245 is a FAD-dependent monooxygenase, these results suggest that NRRL3_10245 converts 3,5-DHB to 2,3,5-THB. We therefore refer to NRRL3_10245 as 3,5-dihydroxybenzoate 2-monooxygenase and its encoding gene *dboA*.

Bacterial gentisate 1,2-dioxygenases catalyze the oxidation of gentisate to maleylpyruvate. While the name suggests specificity for gentisate, studies have shown that these enzymes are not specific to gentisate but often exhibit broad substrate specificity, allowing them to act on other hydroxybenzoates that are structurally similar, albeit with significantly varying affinity and specificity (Hintner *et al.*, 2004; Subbotina *et al.*, 2021). This reflects the functional promiscuity commonly observed in bacterial gentisate 1,2-dioxygenases (Matera *et al.*, 2008; Khersonsky & Tawfik, 2010; Subbotina *et al.*, 2021). Additionally, it demonstrates the wide functional and structural diversity of gentisate 1,2-dioxygenases among bacteria. Some show

narrow substrate specificity, while others, like the gentisate 1,2-dioxygenases from *Pseudaminobacter salicylatoxidans*, display a broader activity profile (Matera *et al.*, 2008; Ferraroni *et al.*, 2013).

The accumulation of 2,3,5-THB in $\Delta NRRL3_10244$ strains suggests that *NRRL3_10244* encodes an enzyme crucial for the downstream conversion of 2,3,5-THB in the resveratrol catabolic pathway. Although *NRRL3_10244* is a paralogue of the *A. niger* gentisate 1,2-dioxygenase GdoA (*NRRL3_05236*), we have shown by gene deletion analysis that *NRRL3_10244* is not involved in gentisate catabolism (Chapter 2). These results imply that the physiological role of *NRRL3_10244* has evolved beyond gentisate catabolism, aligning with the degradation of other benzoates that require a 1,2-dioxygenase. If *NRRL3_10244* cleaves 2,3,5-THB at the 1,2-position, the product would be hydroxymaleylpyruvate. When extracts of *B. brevis* were used to hydrolyze 2,3,5-THB, oxaloacetate and pyruvate were observed. Crawford and Perkins argued that oxaloacetate could only arise if the product of 2,3,5-THB was hydroxymaleylpyruvate. Putting the two convergent predictions together suggests that *NRRL3_10244* is a 2,3,5-trihydroxybenzoate 1,2-dioxygenase, and we therefore name its encoding gene as *tdoA*.

3.3.6 $\Delta NRRL3_10248$ results in severe growth impairment.

NRRL3_10248 encodes a predicted fumarylacetoacetatase and is predicted to catalyze the final step of 3,5-dihydroxybenzaldehyde catabolism to the TCA intermediates oxaloacetate and pyruvate. The prediction expects hydroxymaleylpyruvate to interconvert with oxaloacetylpyruvate spontaneously, and *NRRL3_10248* hydrolyzes oxaloacetylpyruvate into oxaloacetate and pyruvate. To determine its function, we deleted *NRRL3_10248*. The resulting mutants exhibited significantly reduced growth or failed to grow on resveratrol, 3,5-dihydroxybenzaldehyde, and 3,5-DHB, but showed growth rates equal to the parent strain on fructose, 4-hydroxybenzaldehyde, and 4-hydroxybenzoate (Figures 3.3 and 3.5).

As with the other pathway mutants, $\Delta NRRL3_10248$ is expected to accumulate the catalytic product(s) of 2,3,5-THB, hydroxymaleylpyruvate and oxaloacetylpyruvate. Analysis by LC-MS of $\Delta NRRL3_10248$ cultivated on resveratrol as the sole carbon source revealed the accumulation

of a metabolite with an m/z of 201.0603, which was not detected in the parent strain (Figure 3.14). As well, the rate of resveratrol utilization was observed to be slower in the mutant strain $\Delta NRRL3_10248$ than in the parent strain (Figure 3.15).

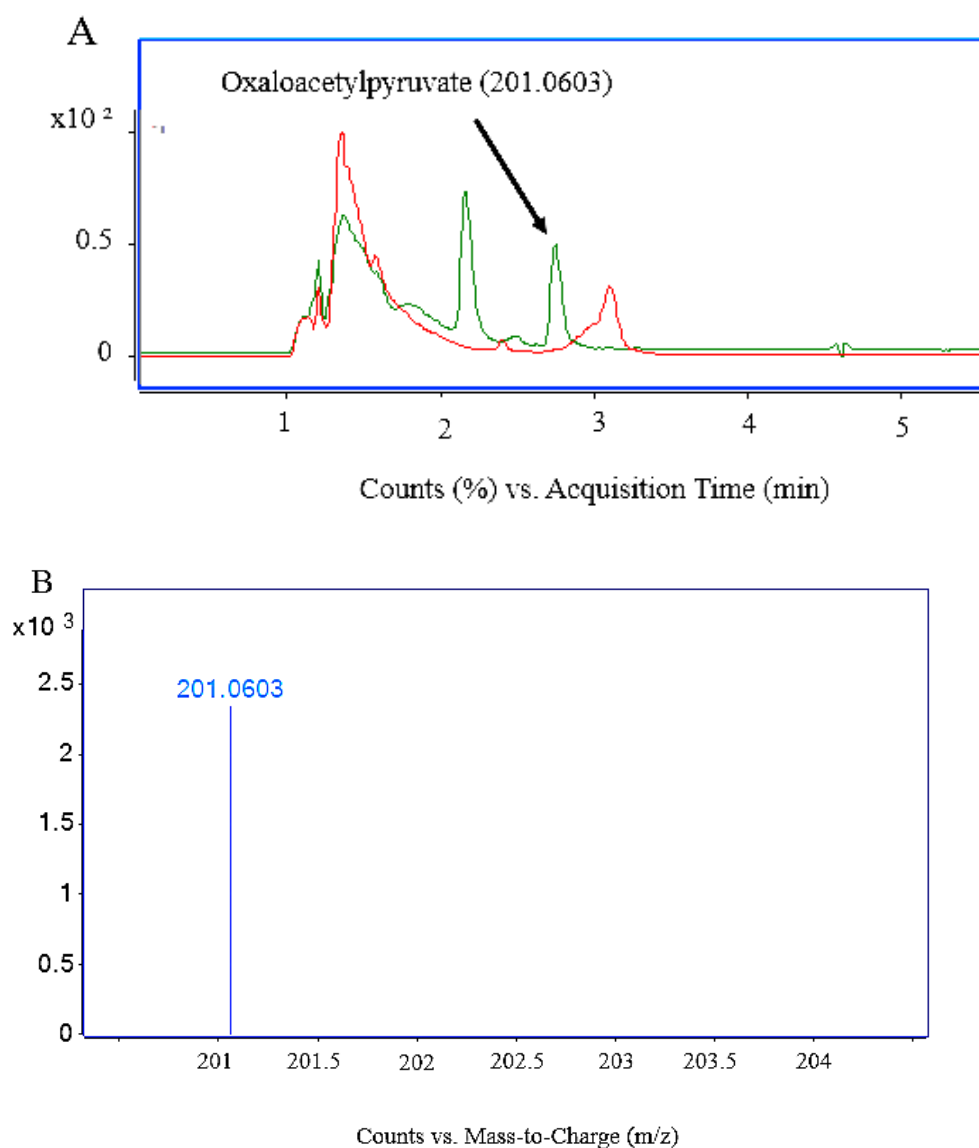


Figure 3.14 Accumulation of a Low-Intensity Intermediate in the $\Delta ophA$ ($\Delta NRRL3_10248$).

Total ion chromatograms (TIC) showing a peak at m/z 201.0603 detected only in cultures of the mutant strain $\Delta ophA$ (green peak) grown on resveratrol but absent in the *A. niger* parent strain

(red peak). The weak MS signal suggests accumulation of a transient or low-abundance intermediate, consistent with the predicted ring-cleavage product, oxaloacetylpyruvate.

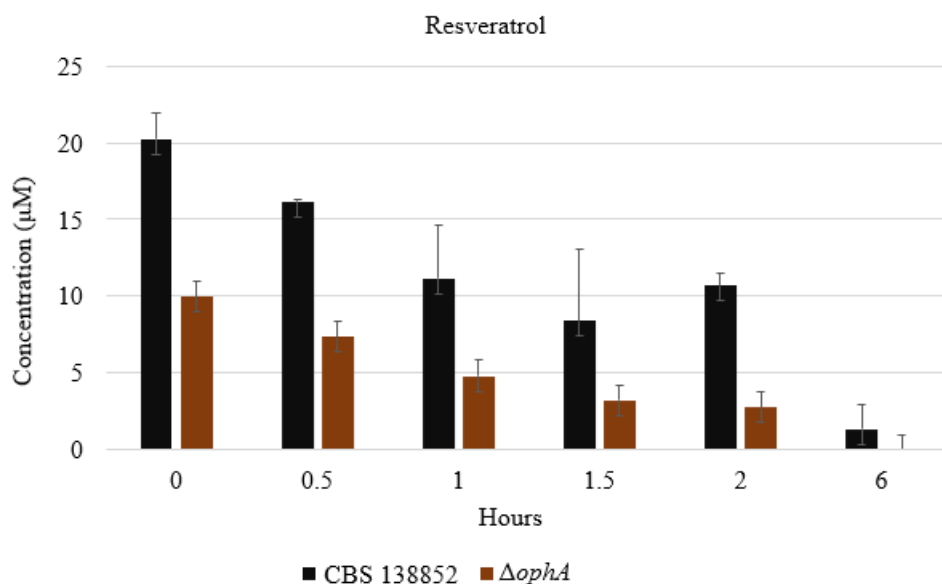


Figure 3.15 LC-MS analysis of resveratrol utilization. Resveratrol consumption in the *A. niger* parent strain and mutant $\Delta ophA$ (NRRL3_10248) strain.

However, the signal intensity of this peak was low, suggesting that the intermediate was present at a minimal concentration. As a result, MS/MS analysis did not produce a fragmentation spectrum. While this prevented direct confirmation of its structure, the observed m/z is consistent with either hydroxymaleylpyruvate or oxaloacetylpyruvate, both of which share the same molecular formula ($C_7H_5O_8^-$) and exact mass. Thus, the compound observed in the $\Delta NRRL3_10248$ likely corresponds to one of these intermediates, but the specific identity could not be confirmed.

Similar challenges have been reported in other pathways. For example, in *Aminobacter* sp., the degradation of the groundwater pollutant, 2,6-dichlorobenzamide, via an extradiol dioxygenase resulted in a transient ring fission product that was difficult to detect, potentially due to spontaneous abiotic conversion (Raes *et al.*, 2019). Likewise, in *Bacillus brevis*, the degradation of 3,5-DHB to 2,3,5-THB led to the formation of an unstable ring fission product that could not

be detected (Crawford & Perkins, 1978). This transient nature of the intermediate suggests that, like the $\Delta NRRL3_10248$ mutant observed in this study, any potential accumulation of the ring fission product may be too ephemeral or at a concentration below the limit of detection of the LC-MS technique used. Given these precedents and the detectable oxaloacetate and pyruvate as the end-products of 3,5-DHB (Crawford & Perkins, 1978), it is reasonable to conclude that $\Delta NRRL3_10248$, designated as *ophA*, encodes oxaloacetylpyruvate hydrolase. However, since hydroxymaleylpyruvate and oxaloacetylpyruvate have identical masses and cannot be differentiated by MS data alone, future experiments employing targeted MS/MS analysis will be required to confirm the exact identity of the accumulated intermediate.

3.3.7 $\Delta NRRL3_10246$ encodes the regulator of the resveratrol gene cluster

NRRL3_10246 contains a fungal-specific transcription factor domain (PFam04082), suggesting that it is a transcription factor. Co-localized with predicted resveratrol pathway genes, its deletion ($\Delta NRRL3_10246$) resulted in an impairment of growth on resveratrol. This suggests that NRRL3_10246 acts as a regulator required for the induction of catabolic genes within the cluster, including those encoding ring-cleavage and hydroxylation enzymes. However, growth was unaffected on 4-hydroxybenzaldehyde and 4-hydroxybenzoate, indicating the non-involvement of NRRL3_10246 in the catabolism of these compounds (Figures 3.3, 3.5). Comparable transcriptional regulators have been described in *A. niger* secondary metabolic clusters, where they coordinate substrate-responsive expression of pathway genes (Semper *et al.*, 2025). Together, these findings support the role of NRRL3_10246 as a cluster-specific transcriptional activator essential for the efficient utilization of resveratrol, and we designate the gene *rvtR*.

3.3.8 Conversion of 4-hydroxybenzoate to protocatechuate

The two aldehydes, 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde, generated by the catalysis of resveratrol by RcoA, are converted to their respective benzoates by VdhA (Sections 3.3.2 and 3.3.3). The degradation of 4-hydroxybenzoate (4-DHB) has been hypothesized to go through the protocatechuate (3,4-DHB) pathway (Kurt *et al.*, 2018). Lubbers *et al.* (2019) showed that in *A. niger*, the enzyme *p*-hydroxybenzoate *m*-hydroxylase (*PhhA*, NRRL3_8551) converts 4-HB to 3,4-DHB. Deletion of *phhA* resulted in the loss of growth on *p*-hydroxybenzoate but did not affect utilization of 3-hydroxybenzoate, demonstrating that *phhA* is specific to the 4-

HB to 3,4-DHB step, thereby linking the 4-hydroxybenzaldehyde part of the resveratrol pathway to 3,4-DHB.

3.3.9 Phenotypes of 3,4-DHB mutants on 4-hydroxybenzaldehyde and 4-hydroxybenzoate

The catabolic pathway of protocatechuate (3,4-DHB) has been characterized previously, and mutants in each step of this pathway are available (Sgro *et al.*, 2023). Figure 3.5 showed that *A. niger* mutants defective in the 3,4-DHB pathway ($\Delta prcA$, $\Delta cmcA$, $\Delta chdA$, $\Delta kstA$ and $\Delta kctA$) exhibited complete loss of growth on 4-hydroxybenzaldehyde and 4-hydroxybenzoate, but not on 3,5-DHB. These results provide direct evidence that the 4-hydroxybenzaldehyde part of the resveratrol catabolic pathway is channelled through the 3,4-DHB pathway.

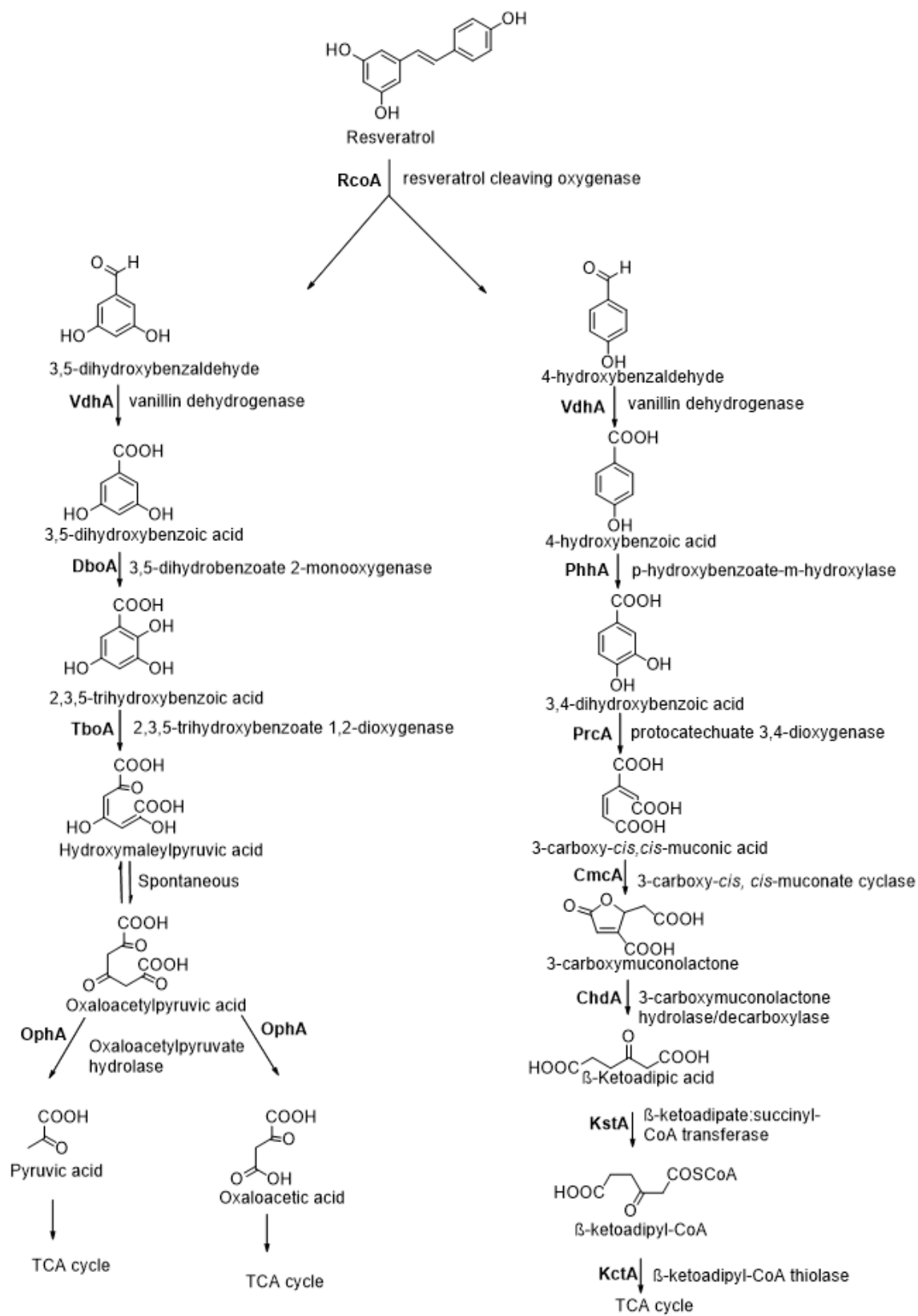


Figure 3.16 Pathway for the degradation of resveratrol. *Aspergillus niger* genes encoding enzymes in the pathway are assigned based on experimental evidence.

3.4 Conclusion

In this study, we report the identification of a gene cluster in *Aspergillus niger* (NRRL3_10244–10248) encoding the enzymes required for the degradation of resveratrol and one of its two cleavage products, 3,5-dihydroxybenzaldehyde. The first reaction in this pathway is catalyzed by RcoA (NRRL3_10247), a resveratrol-cleaving dioxygenase that catalyzes the oxidative cleavage of resveratrol to yield 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde. These aldehydes are then oxidized to their corresponding benzoates, 3,5-dihydroxybenzoate and 4-hydroxybenzoate by VdhA, a benzaldehyde dehydrogenase previously shown to act on various aromatic aldehydes. While the catabolic route for 4-hydroxybenzoate via the protocatechuate branch of the β -ketoadipate pathway has been characterized, we describe here the fate of resveratrol-derived 3,5-dihydroxybenzoate degradation in *A. niger*. The next step involves the hydroxylation of 3,5-dihydroxybenzoate by DboA (NRRL3_10245), a monooxygenase. This enzyme introduces a hydroxyl group to form 2,3,5-trihydroxybenzoate, as verified by LC-MS. Following this step, 2,3,5-trihydroxybenzoate 1,2-dioxygenase TboA (NRRL3_10244), a ring-cleaving dioxygenase, breaks open the aromatic ring of 2,3,5-trihydroxybenzoate to form hydroxymaleylpyruvate. This unstable intermediate is predicted to tautomerize to oxaloacetylpyruvate, which is subsequently hydrolyzed by oxaloacetylpyruvate hydrolase OphA (NRRL3_10248) to produce oxaloacetate and pyruvate, thereby linking the pathway to the TCA cycle.

Together with the demonstrated involvement of the protocatechuate pathway, these findings reveal a complete fungal pathway for resveratrol degradation (Figure 3.16). This pathway demonstrates how *A. niger* converts plant-derived stilbenes into central metabolites, expanding our understanding of fungal aromatic metabolism and its potential biotechnological applications.

3.5 Materials and Methods

3.5.1 Chemicals

Resveratrol, 4-hydroxybenzaldehyde, 3,5-dihydroxybenzaldehyde, and 3,5-dihydroxybenzoic acid were obtained from Fisher Scientific (Ottawa, ON, Canada), and 2,3,5-trihydroxybenzoic acid was purchased from Dalton Pharma Services (Toronto, ON, Canada). All chemicals used in this study were of the highest commercially available purity and used as received with no further purification.

3.5.2 Strains, media and conditions of growth

This study utilized *Aspergillus niger* strain CBS 138852 as the parental strain for generating mutant strains through gene deletion (Table 3.1). This strain is characterized by being auxotrophic for uridine, exhibiting short conidiophores, and carrying a *kusA* gene deletion, which enhances homologous recombination efficiency (Alazi *et al.*, 2016).

Table 3.1 *A. niger* strains used in this study.

Strain	Genotype	Source
N593	NRRL3 <i>cspA1</i> Δ <i>pyrG</i>	(Debets & Bos, 1986)
CBS 138852	N593 Δ <i>kusA</i>	(Alazi <i>et al.</i> , 2016)
IBO_17	CBS 138852 Δ NRRL3_10244	This study
IBO_18	CBS 138852 Δ NRRL3_10245	This study
IBO_19	CBS 138852 Δ NRRL3_10246	This study
IBO_20	CBS 138852 Δ NRRL3_10247	This study
IBO_21	CBS 138852 Δ NRRL3_10248	This study
IBO_22	CBS 138852 Δ NRRL3_03887	This study
IBO_1	CBS 138852 Δ NRRL3_05234-05237	This study
IBO_2	CBS 138852 Δ NRRL3_10244-10250	This study

Fungal spores (1×10^6 spores/mL) for protoplastation were inoculated into 200 mL of complete medium (minimal medium supplemented with 10 mM uridine, 1 g/L casamino acids, and 5 g/L yeast extract). Protoplast preparation followed established protocols. After protoplast generation, transformants were cultured on selective minimal medium plates at 30°C for 5 days (Hill & Kafer, 2001).

For genomic DNA extraction and PCR screening, 2-3 colonies from each transformation plate were isolated, purified, and inoculated into 250 μ L of complete medium, then incubated at 30°C for 17 hours. Following overnight incubation, DNA extraction from mycelia mats for PCR-based analyses was conducted following established protocols (Bellemare *et al.*, 2018).

Growth profile experiments were conducted by spotting 2 μ L of saline/Tween containing 1000 freshly harvested spores onto minimal medium plates containing 0.5% fructose or various aromatic compounds as the sole carbon source. All plasmids generated in this study were propagated and maintained in *Escherichia coli* strain DH5 α .

For metabolite analysis, transfer culture experiments were conducted. Fungal spores at a concentration of 2×10^6 spores/mL were initially grown in 200 mL complete medium and incubated overnight at 30°C with agitation at 220 rpm. Then, 250 mg of washed and dried mycelia were transferred to 25 mL minimal medium with 200 µM resveratrol as the sole carbon source and cultivated for 6 hours at 30°C with shaking at 220 rpm.

3.5.3 Guide sequence selection and validation

We used Geneious R9.1 software (<http://www.geneious.com>) (Kearse *et al.*, 2012) to identify 20 bp guide sequences against the *A. niger* NRRL3 genome and confirm their specificity, with no off-target sites identified.

3.5.4 Construction of gene deletion cassettes using CRISPR/Cas9

CRISPR/Cas9 technology was employed to generate gene deletion mutants, as previously described (Song *et al.*, 2018). The guide RNA expression cassettes were cloned into the ANEp8-Cas9 plasmid using ligation-independent cloning as previously described (Song *et al.*, 2018). Co-transformation of *A. niger* with CRISPR plasmids and 60 or 90-base gene-editing oligonucleotides, with 30- or 45-base homology arms, enabled precise deletion mutant generation through targeted chromosomal break repair. Target sequences for CRISPR, primers, and oligos used in this study are listed in the legends of the verification Figures S3.1–S3.8.

3.5.5 Transformation, mutant purification, and screening protocol

A. niger protoplasts were generated using young hyphae as previously described (Debets & Bos, 1986). Transformations were performed as described (Meyer *et al.*, 2010) using 1.5 µg CRISPR plasmid DNA and 1 nmol gene-editing oligonucleotide. For each gene, two or more independent transformants were isolated for phenotypic analysis. PCR amplification and agarose gel electrophoresis (Figs. S2-S9) verified mutations in the transformants using primers listed in the figure descriptions.

3.5.6 Identification of an additional homologue from related fungal species

Previous research in bacteria and fungi showed that aldehyde dehydrogenase is necessary for the biotransformation of resveratrol ring cleavage products (Kurt *et al.*, 2018; Wang *et al.*, 2019). While none of the predicted genes within the cluster (NRRL3_10244–NRRL3_10250) is annotated as an aldehyde dehydrogenase, we identified a gene cluster neighbouring a putative aldehyde dehydrogenase in related *Aspergillus* species. We used the in-cluster aldehyde dehydrogenase of *A. brasiliensis*, *A. fumigatus*, and *A. oryzae* (Figure 3.2) to perform BLASTP searches against the *A. niger* NRRL3 genome. Each search returned as the top hit a previously characterized vanillin dehydrogenase *VdhA* (NRRL3_03887) (Lubbers *et al.*, 2024), with sequence identity > 50% and this was therefore selected as a candidate aldehyde dehydrogenase for further study.

3.6 Metabolite profiling by liquid chromatography-mass spectrometry

3.6.1 Sample preparation

Duplicate cultures of parental and mutant strains were transferred to minimal medium containing resveratrol. Samples were collected at various time points and transferred to microcentrifuge tubes. The cells were pelleted by centrifugation at 13,300 x g for 30 minutes. Supernatants were transferred to new tubes and subjected to methanol precipitation at –20°C for 10 minutes. Samples were centrifuged at 13,300 x g for 30 minutes to remove precipitated proteins. Supernatants were transferred to new tubes, and an equal volume of 0.1% formic acid was added to enhance chromatographic separation.

3.6.2 Liquid chromatography-mass spectrometry (LC-MS) analysis

Electrospray LC-MS analysis was performed using an Agilent 6560 Ion Mobility Q-TOF and 1290 Infinity II LC-MS system (Agilent Technologies, Santa Clara, CA). The scan range was set from 100 to 1400 m/z. Reversed-phase liquid chromatography was performed using a Synergy 4 µm Hydro-RP 80 Å, 150 × 2.00 mm column (Phenomenex, Torrance, CA).

3.7 Data analysis

Data analysis was performed using Agilent MassHunter software. Chromatograms and mass spectra were evaluated for peak intensity, retention time, and mass accuracy.

3.7.1 Quantification methodology

Compound concentrations were quantified using a standard curve approach. LC-MS analysis of calibrated standards with known concentrations generated a plot of peak area versus concentration. The resulting linear regression curve was used to determine concentrations in experimental samples.

3.8 Data availability

All data are contained within the manuscript.

3.9 Funding and additional information

This work was supported by the Biocatalysis Strategic Network of the Natural Sciences and Engineering Research Council of Canada.

3.10 Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

3.11 Supplementary Figures

Figure S3.1-S3.8 PCR verification of deletions in resveratrol pathway genes

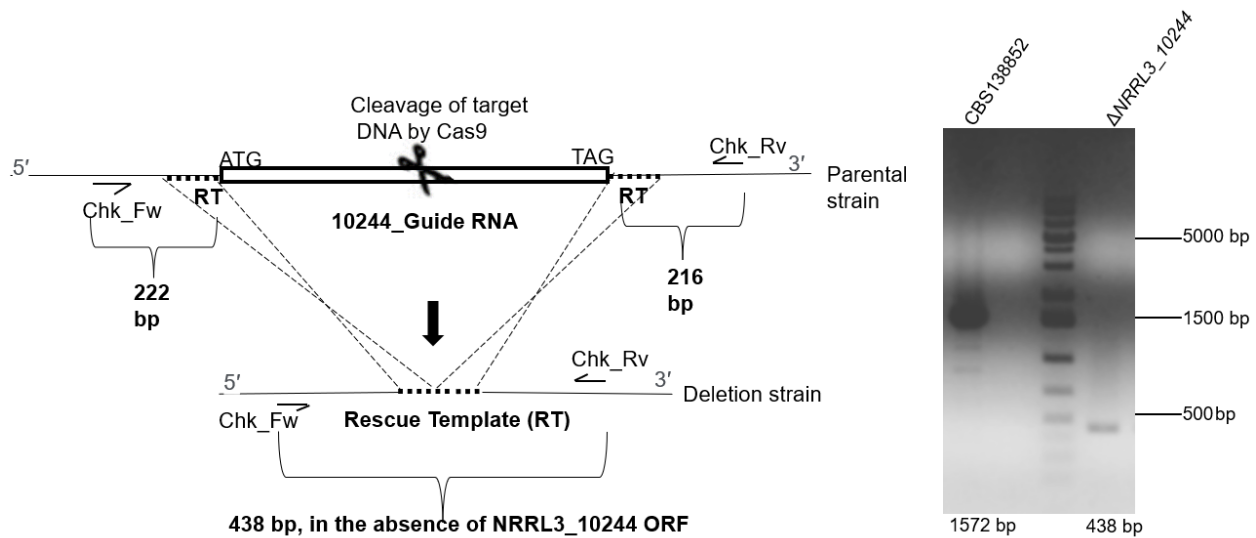


Figure S3.1 Diagram of mutant generation and verification of Δ NRRL3_10244.

CRISPR/Cas9 was used to create a double-stranded break with the target sequence GTACAAGTATCGGCATATTG. The rescue template (RT) gene-editing oligonucleotide-sequence GCTAGATCTCGACACTTTCGCACTCCCAT-AACCTTGAACTGCCGATGATGTCTGAAGGA on either side of the ORF was used to repair the deletion. Primers around the deletion (10244Fw: GGTCCAAAACACGTTACTAT and 10244 Rv: CTCTGTGGATCCTTGTGGG) were used for PCR verification, resulting in a 438 bp mutant. Expected band sizes for mutant and parent strains are shown below each PCR product.

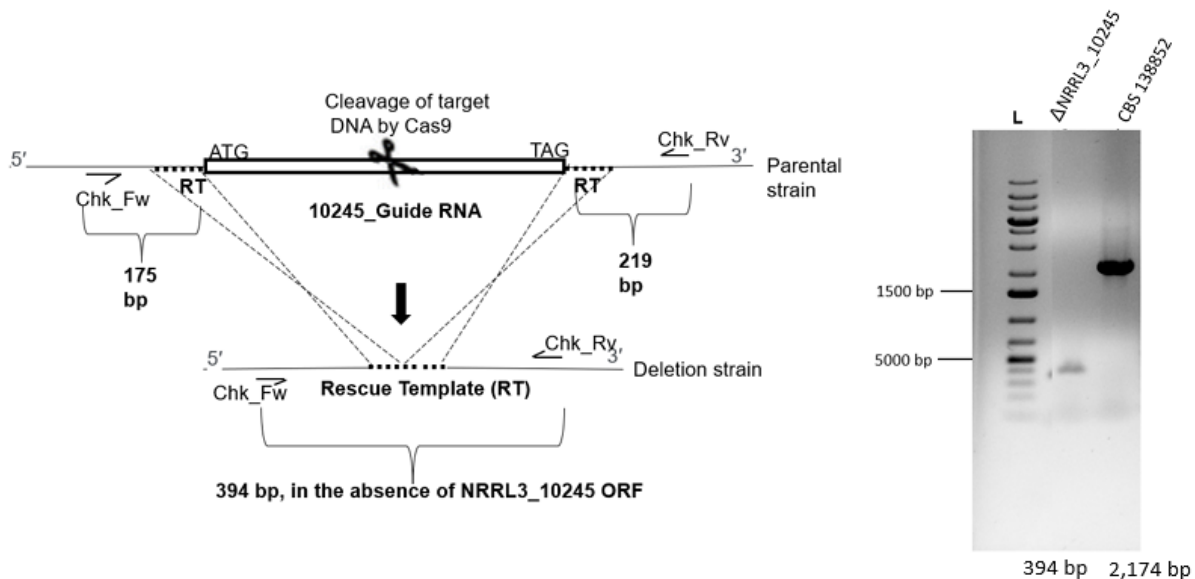


Figure S3.2 Diagram of mutant generation and verification of Δ NRRL3_10245.

CRISPR/Cas9 was used to create a double-stranded break with the target sequence TCAGTCATAGTCCAAACCAG. The rescue template (RT) gene-editing oligonucleotide-sequence ACGGCAGACCTCAATACCACGAATATTGGA - AATCTAGATTTGCGCAGGCTGTTATGCAGA on either side of the ORF was used to repair the deletion. Primers around the deletion (10245_Fw: CGTGTCGTTACTACCCTAC and 10245_Rv: CGTGATCTACTTACCCCGG) were used for PCR verification, resulting in a 394 bp mutant. Expected band sizes for mutant and parent strains are shown below each PCR product.

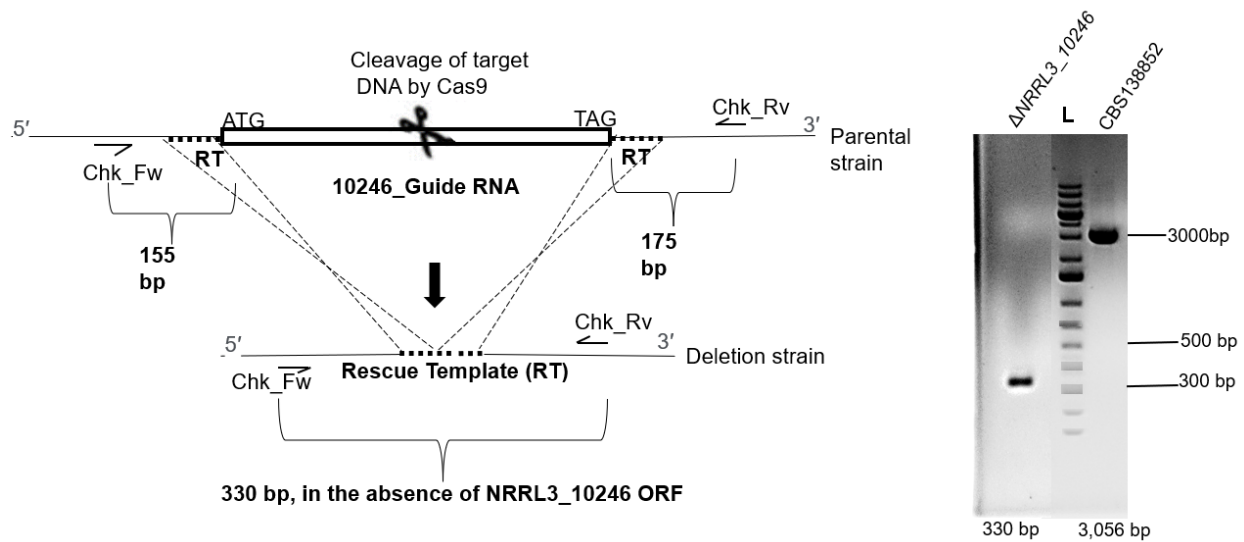


Figure S3.3 Diagram of mutant generation and verification of Δ NRRL3_10246.

CRISPR/Cas9 was used to create a double-stranded break with the target sequence CGTAAAATCTCTCCCTTGCA. The rescue template (RT) gene-editing oligonucleotide-sequence CGATCTGCTACTTTTACCCGCTAGACCAAA-GGAGGGGGCGCCCCGGGTGGGGGGATTGGA on either side of the ORF was used to repair the deletion. Primers around the deletion (10246_Fw: CGTGATTGGCCGCCTTCTC and 10246_Rv: GCTTGGTATCTAGGCGAGTC) were used for PCR verification, resulting in a 330 bp mutant. Expected band sizes for mutant and parent strains are shown below each PCR product.

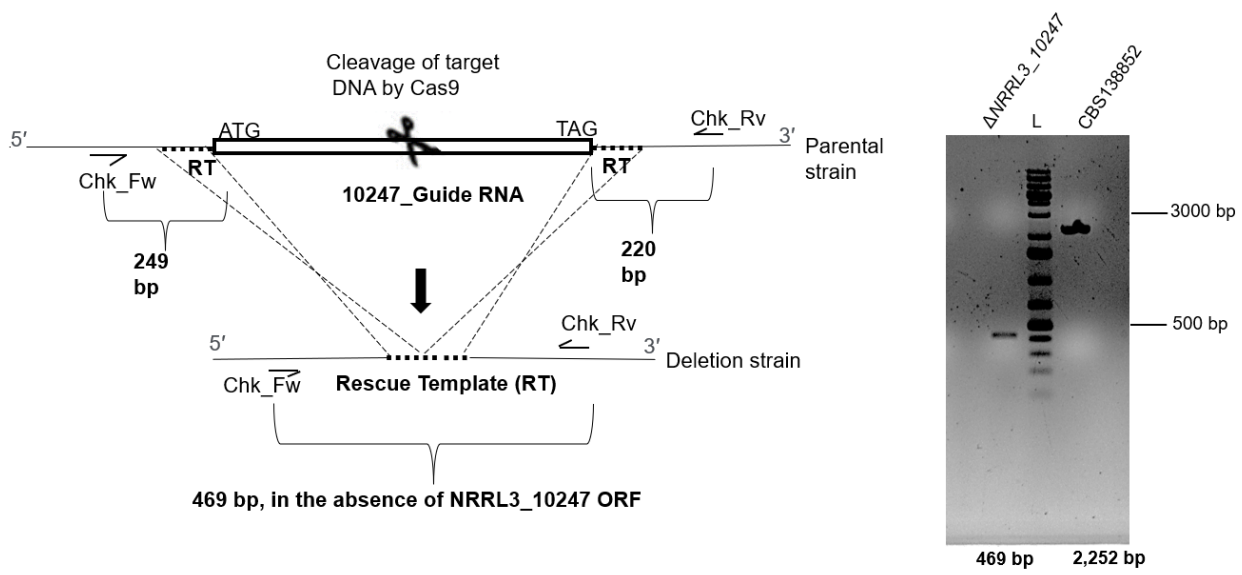


Figure S3.4 Diagram of mutant generation and verification of Δ NRRL3_10247.

CRISPR/Cas9 was used to create a double-stranded break with the target sequence AACCCTGAATCTGGAGGTCT. The rescue template (RT) gene-editing oligonucleotide-sequence CCTTTAGTAGGAAATACACATATTGCAGCC-TAGGGAGGAGTAGTTTTGTGGATTGCATGA on either side of the ORF was used to repair the deletion. Primers around the deletion (10247_Fw: GCTTCACGACCAGGTAGTCAC and 10247_Rv: CTTTATGATAGGGCGCATGC) were used for PCR verification, resulting in a 469 bp mutant. Expected band sizes for mutant and parent strains are shown below each PCR product.

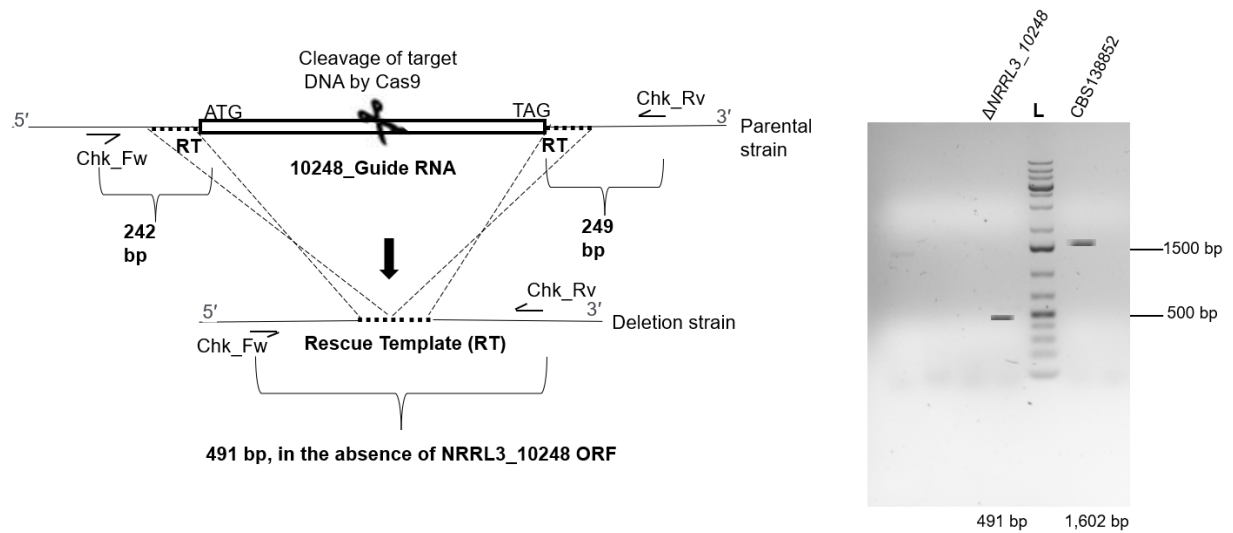


Figure S3.5 Diagram of mutant generation and verification of Δ NRRL3_10248. CRISPR/Cas9 was used to create a double-stranded break with the target sequence GATCGCACAGAATGACCAGG. The rescue template (RT) gene-editing oligonucleotide-sequence TAAGCTGTCATACCTTAAAGAACGCCTGTT-AACGGGTCGAACTGTTGACGATAAAGAATC on either side of the ORF was used to repair the deletion. Primers around the deletion (10248_Fw: GCAAAAGGTGGACCGCAAC and 10248_Rv: GCTCGGTGTCCTCCTTAAATC) were used for PCR verification, resulting in a 491 bp mutant. Expected band sizes for mutant and parent strains are shown below each PCR product.

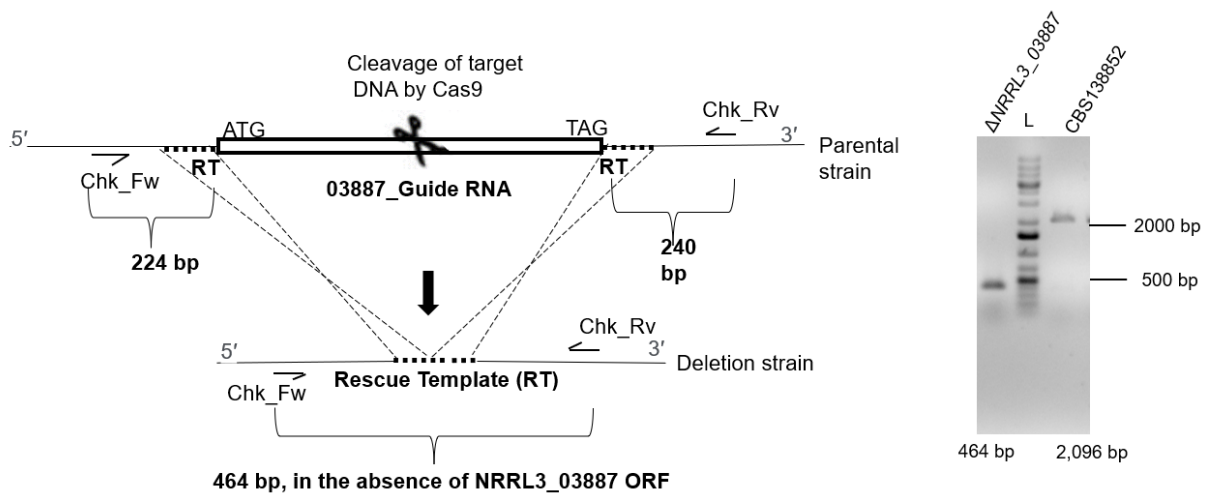


Figure S3.6 Diagram of mutant generation and verification of Δ NRRL3_03887.

CRISPR/Cas9 was used to create a double-stranded break with the target sequence CCAAACGCATTACTTCCTCG. The rescue template (RT) gene-editing oligonucleotide-sequence ACAATCACACACTCCCCGGACATACCAAG-TGTGTATGTAGCCAAATAAGTTGCGTAATC on either side of the ORF was used to repair the deletion. Primers around the deletion (03887_Fw: CGAACAAGGACATGATGGGG and 03887_Rv: TACATGAAAAGAGATATTAGGCATGATCTG) were used for PCR verification, resulting in a 464 bp mutant. Expected band sizes for mutant and parent strains are shown below each PCR product.

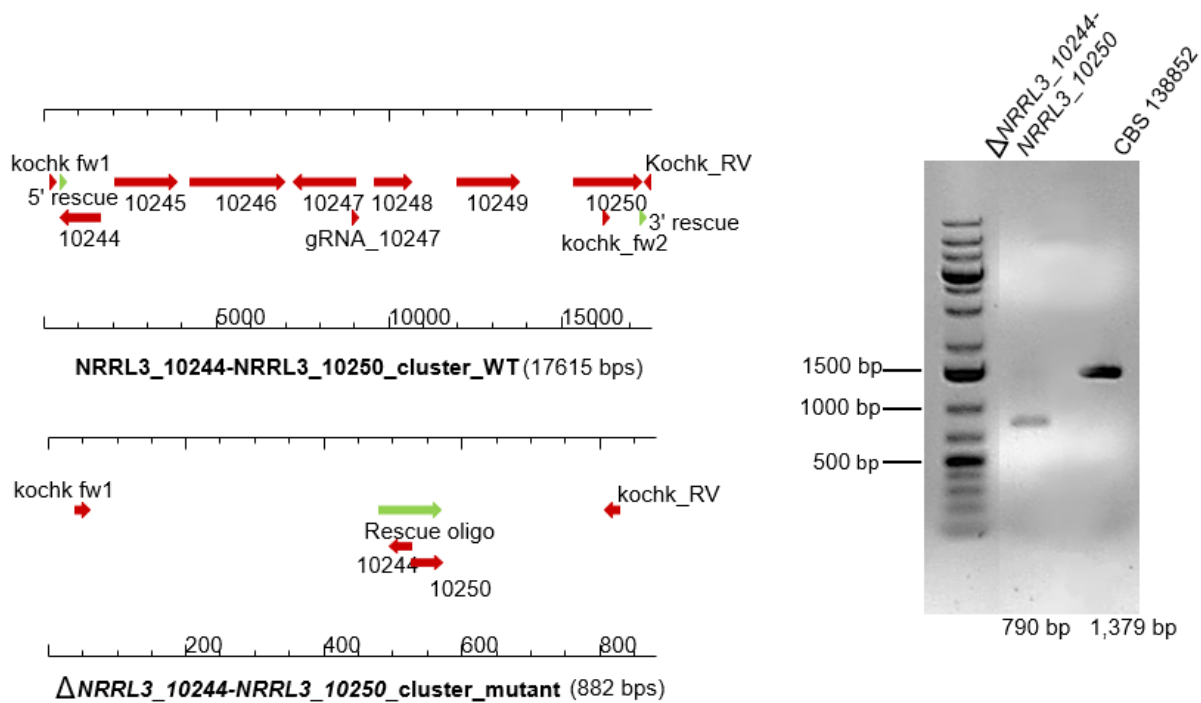


Figure S3.7 PCR verification of Δ NRRL3_10244-NRRL3_10250.

The target sequence for the CRISPR/Cas9 gRNA was AACCCTGAATCTGGAGGTCT. The rescue template oligonucleotide sequence-
GGCAGTTCAAGGTTCTACTGGGACACCAGCGTCGCCAGATCCGTAT-
CTGGGGGTGAAGTTTGGGTTGTGGAATGCTACGAATCCTTATTG with complementary
sequences on either side of the intended deletion, was used to repair the double-stranded break.
Primers flanking the deletion (Δ NRRL3_10244-NRRL3_10250 Fw1:
CACTACTCTCCAGGGCTGGATC: Δ NRRL3_10244-NRRL3_10250 Fw2:
CCAGCTAACGCGGTCTAGTTG: Δ NRRL3_10244-NRRL3_10250_Rv:
CACTACTCTCCAGGGCTGGATC were used for PCR verification, resulting in a 790 bp
fragment in the mutant compared to the 1,379 bp in the parental strain.

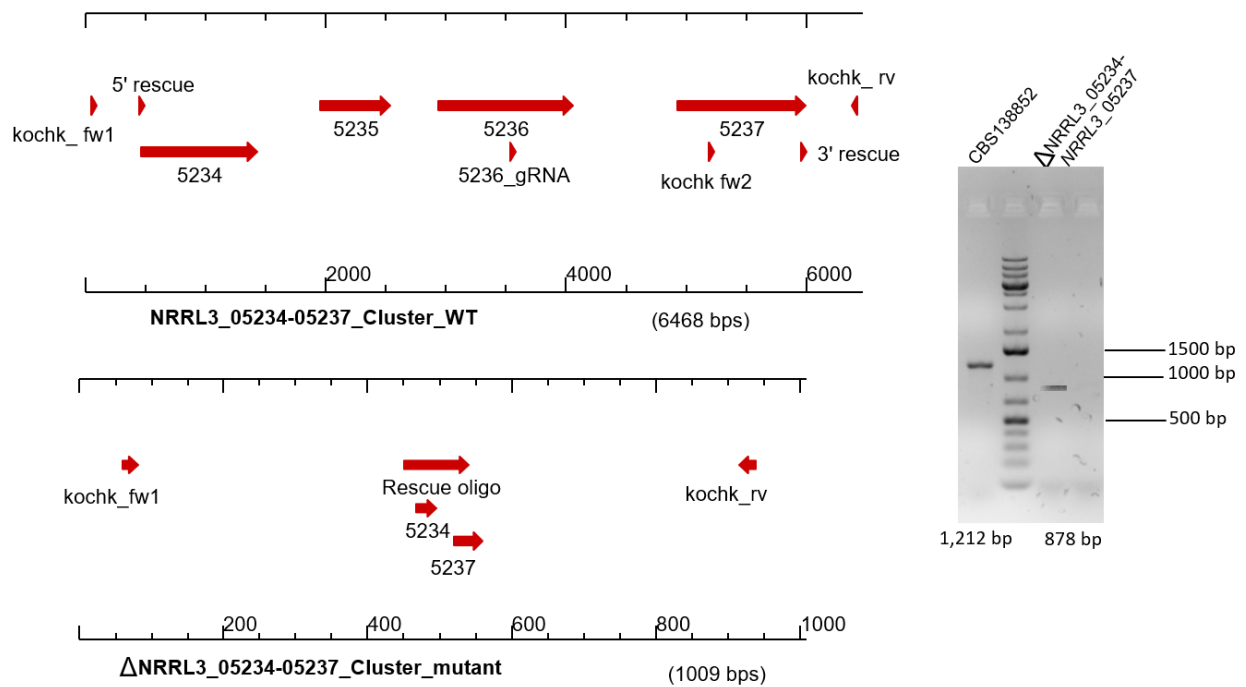


Figure S3.8 Diagram of mutant generation and verification of Δ NRRL3_05234-05237. CRISPR/Cas9 was used to create a double-stranded break with the target sequence CGACGGATACATCGGCACCT. The rescue template (RT) gene-editing oligonucleotide-sequence GACTTGCCTGCCCATCATGGTCCAAGCAAAAGCCTTCATTGTCCC-AGGGGCTGGGTGCGACGAGGAACGTTATGGTTTTTGAGTAGGGAG on either side of the ORF was used to repair the deletion. Primers around the deletion (Δ NRRL3_05234-05237 Check Fw1: CCTGGAGGAAGACCTACCTGTG, Δ NRRL3_05234-05237 Check Fw2: GTGCAAGACTCAGTTGGTACCAG and Δ NRRL3_05234-05237 Rv: GATTAAGACGGTGGAGGCGGATAG) were used for PCR verification, resulting in an 878 bp mutant. Expected band sizes for mutant and parent strains are shown below each PCR product.

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Chapter 4: Comprehensive insights into the gentisate (2,5-DHB) and resveratrol catabolic pathways in *Aspergillus niger*: Conclusions and future direction

Aromatic compound degradation is an essential metabolic process in fungi, influencing ecological roles and industrial applications. This study provides the first in-depth functional characterization of gentisate and resveratrol catabolic pathways in *A. niger*, elucidating the genes encoding enzymes involved in their catabolism.

4.1 Gentisate catabolic pathway

Using a combination of comparative genomics, transcriptomics, and gene deletion experiments, this study identified and functionally validated *in vivo* the genes encoding enzymes involved in the gentisate catabolic pathway. The *gdoA* was found to encode gentisate 1,2-dioxygenase, catalyzing the cleavage of gentisate into maleylpyruvate. The *fphA* encodes fumarylpyruvate hydrolase, which catalyzes the hydrolysis of fumarylpyruvate into fumarate and pyruvate. The deletion of these genes resulted in severe growth defects on gentisate, thus confirming their indispensable roles in the pathway.

A notable observation in this study was that the *miaA* gene product facilitates the conversion of maleylpyruvate to fumarylpyruvate. In contrast to bacteria, the *miaA* shares no sequence or structural similarity with previously characterized maleylpyruvate isomerase, highlighting a unique evolutionary adaptation in *A. niger*. The upregulation of *miaA* during growth on gentisate and the accumulation of maleylpyruvate in the Δ *miaA* deletion mutant further validated its functional role. This finding suggests that *A. niger* employs a distinct enzymatic mechanism for aromatic compound degradation, underscoring the diversity of fungal metabolism.

4.2 Resveratrol catabolic pathway

The resveratrol catabolic pathway in *A. niger* was evaluated via the functional characterization of genes within the *NRRL3_10244–NRRL3_10248* cluster. The *NRRL3_10247* (*rcoA*) was identified as encoding resveratrol cleavage dioxygenase, responsible for breaking the stilbene backbone of resveratrol to yield aldehyde intermediates. The deletion of this gene resulted

in the accumulation of resveratrol in the extracellular medium and the consequent impairment of fungal growth, thus confirming its pivotal role in resveratrol catabolism.

Another enzyme, previously characterized, NRRL3_03887 (*vdhA*), was found to encode benzaldehyde dehydrogenase, catalyzing the oxidation of resveratrol-derived aldehydes into their respective hydroxybenzoate derivatives. The deletion of $\Delta vd h A$ led to growth impairment on 3,5-dihydroxybenzaldehyde and resulted in its extracellular accumulation, reinforcing its function in aldehyde oxidation. More so, the NRRL3_10245 (*dboA*) was identified as encoding a 3,5-dihydroxybenzoate, necessary for the downstream metabolism of resveratrol-derived 3,5-dihydroxybenzoate. Knockout studies demonstrated a significant growth defect in 3,5-dihydroxybenzoic acid and its accumulation in $\Delta dbo A$, further substantiating its role in the pathway.

Interestingly, NRRL3_10244 (*tdoA*) was initially predicted to function as a gentisate 1,2-dioxygenase based on orthology and sequence similarity. However, genetic and metabolomic analyses revealed that its deletion led to the accumulation of 2,3,5-trihydroxybenzoic acid, indicating an alternative enzymatic function as a 2,3,5-trihydroxybenzoate 1,2-dioxygenase. The functional divergence of *tdoA* from classical gentisate dioxygenases represents a novel adaptation in fungal metabolism.

Furthermore, this study identified the gene NRRL3_10248 (*ophA*) as a critical component in the catabolic pathway of resveratrol-derived aromatic compounds in *Aspergillus niger*. Deletion of $\Delta oph A$ resulted in severe growth defects on resveratrol and 3,5-dihydroxybenzaldehyde. Although the predicted intermediate was detected by LC-MS, its low abundance was below the MS/MS detection threshold, preventing confirmation of its fragmentation pattern. Consequently, it remains uncertain whether the accumulated product corresponds to hydroxymaleylpyruvate or its isomer, oxaloacetylpyruvate.

Future studies should focus on characterizing the enzymatic activity of OphA and the analysis of enzymatic reaction products, and identifying transient intermediates to fully elucidate

its role. Understanding this pathway may support future applications in bioconversion and biodegradation of plant-derived polyphenols.

4.3 Implications and future directions

The findings from this study have broad implications for both fundamental fungal biology and biotechnological applications, especially in the context of lignin-derived aromatic metabolism. The identification of *miaA* as critical for the conversion of maleylpyruvate opens avenues for biochemical and structural studies. Future efforts should focus on elucidating how MiaA facilitates the conversion of maleylpyruvate to fumarylpyruvate and the biochemical context in which it operates.

Biochemical and structural investigations will be particularly important to evaluate the proposed glutathione-dependent cofactor hypothesis for MiaA. Purified recombinant MiaA could be tested for its ability to restore maleylpyruvate isomerization activity in MiaA-deficient cell extracts supplemented with glutathione (GSH). Isomerase activity would be measured by monitoring a shift under a spectrophotometer from 330 nm (when maleylpyruvate is present) to 345 nm (when it turns into fumarylpyruvate). Comparing reactions with and without added MiaA and across varying GSH concentrations would clarify the functional relationship between MiaA and glutathione and confirm whether GSH acts as a cofactor in this process.

To refine pathway assignments and define enzyme functions, further studies should incorporate targeted metabolite profiling using cell-free extracts from $\Delta miaA$ mutants incubated with gentisate. LC–MS or HPLC analysis of these extracts would allow direct detection of maleylpyruvate, thereby confirming the precise step in the pathway that depends on *miaA*. Such investigations will deepen our molecular understanding of aromatic compound metabolism and its potential biotechnological applications.

Furthermore, regulatory mechanisms governing these pathways require more research. Transcriptomic analyses revealed strong induction of pathway genes in response to gentisate, suggesting the involvement of specific transcriptional regulators. Further studies on these regulators and a holistic understanding of the network of interaction will provide a

comprehensive view of how *A. niger* regulates the catabolism of aromatic compounds in response to signals in the environment.

Beyond fundamental research, elucidating these metabolic pathways has significant industrial potential. The engineering of *A. niger* strains with enhanced gentisate and resveratrol degradation capabilities could facilitate bioremediation efforts, particularly in the degradation of lignin-derived aromatic compounds. Additionally, the unique properties of *miaA* and *vdhA* suggest potential for developing hybrid biocatalytic systems where MiaA facilitates the transformation of structurally similar metabolites into value-added products. For example, blocking maleylpyruvate degradation with the $\Delta miaA$ mutation creates a fungal platform that traps this intermediate for biotechnological use. Instead of the wild-type *A. niger* completely mineralizing gentisate to CO₂, the mutant accumulates maleylpyruvate, which can be converted to fumaric acid. Fumarate goes into polyester resins for construction, food acidulants, and biodegradable plastics.

Another promising avenue is the exploration of microbial interactions in environments rich in aromatic compounds. Understanding how *A. niger* cooperates with bacterial species in biodegradation could lead to the development of synthetic microbial consortia optimized for environmental cleanup. Thus, further comparative genomic analyses across filamentous fungi will also provide insights into these pathways' evolutionary conservation and divergence, expanding our understanding of fungal secondary metabolism.

Finally, this study provides a framework for investigating the degradation of structurally related aromatic compounds beyond gentisate and resveratrol. Given the metabolic versatility of *A. niger*, it is likely that additional, yet unidentified pathways exist for degrading a broader range of aromatic substrates. Future studies integrating transcriptomic, proteomic, and metabolomic approaches will be instrumental in uncovering these pathways and expanding their industrial applications.

4.4 Conclusion

This study provides the first comprehensive functional analysis of the gentisate and resveratrol catabolic pathways in *A. niger*. The identification of pathway genes, their functional validation, and the identification of metabolic intermediates have significantly advanced our understanding of the catabolism of aromatic compounds in fungi. Notably, the characterization of *miaA* and the alternative role of *tdoA* introduce previously unrecognized enzymatic functions in fungal secondary metabolism. Future research focusing on enzyme characterization, regulatory mechanisms, metabolic engineering, and alternative pathways will further elucidate the complexities of these catabolic networks and expand their biotechnological applications. By integrating multi-omics approaches and exploring cross-species metabolic interactions, we can continue to uncover new insights into the metabolic potential of *A. niger* and other filamentous fungi, paving the way for innovative applications in bioremediation, industrial biotechnology, and synthetic biology.