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Endogenous pathway dysregulation in *Kluyveromyces marxianus* under nitrogen limitation drives 2-phenylethyl acetate and 2-phenylethanol overproduction

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The aromatic compounds 2-phenylethanol (2-PE) and 2-phenylethyl acetate (2-PEA) are of high industrial relevance due to their distinctive sensory properties and extensive use in food, fragrance, and wine industries. However, their natural production is costly, motivating interest in sustainable microbial alternatives. This study examined the metabolic regulation of *Kluyveromyces marxianus* ITD0090, a non-conventional yeast, under nitrogen-limited conditions for enhanced 2-PE and 2-PEA production. Combined genomic and transcriptomic analyses revealed key regulatory points associated with aromatic biosynthesis. *K. marxianus* ITD0090 showed remarkable metabolic flexibility, favoring 2-PEA over 2-PE. *ARO9* overexpression appeared to compensate for *ARO8* inactivation, while the shikimate pathway remained active despite external L-phenylalanine, supporting *de novo* phenylalanine synthesis. The preferential accumulation of 2-PEA may represent a cellular detoxification strategy. These results emphasize the potential of *K. marxianus* ITD0090 as a robust, non-engineered platform for cost-effective aroma production without genetic modification or expensive supplementation. This work provides novel insights into alternative metabolic strategies in non-conventional yeasts and opens perspectives for optimizing nitrogen- and carbon-regulated pathways to improve the sustainable production of natural flavor compounds.

Keywords 2-PEA, 2-PE, Aroma production, L-phenylalanine, Nitrogen limitation, Volatile compounds

The aromatic compounds 2-phenylethanol (2-PE) and 2-phenylacetate (2-PEA) are volatile metabolites of great industrial interest due to their unique organoleptic characteristics, which include sweet, fruity, and floral aromas, similar to rose petals. These compounds are used in the oenological, fragrance, and food industries, where they contribute significantly to the sensory profile of fermented products[1–3]. Despite its high commercial value, natural 2-PE is expensive to produce, with a price tag of approximately \$1000/kg, which has led the industry to rely on chemical synthesis from benzene or styrene at a significantly lower cost (\$5/kg)[4]. At the same time,

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there is a growing interest in natural, bio-based products as sustainable alternatives to synthetic chemicals, with the aim of reducing environmental impact. This global demand has motivated the search for biotechnological alternatives for sustainable and natural production.

The global flavor and fragrance market, which includes compounds, such as 2-PE and 2-PEA, reached a value of $28.5 billion in 2022 and is expected to grow at a Compound Annual Growth Rate (CAGR) of 5.3% between 2023 and 2030, driven by the increasing demand for natural and organic products in the food, cosmetics, and pharmaceutical industries[5]. The fragrance sector alone represented 35% of this market, underscoring the economic importance of efficient and natural aroma compound production systems[5].

Kluyveromyces marxianus is a non-conventional yeast that has emerged as a promising platform for the biosynthesis of aromatic compounds. Beyond aroma production, this yeast has also been reported as a host for the synthesis of other functional bioproducts, such as antioxidant exopolysaccharides obtained under optimized fermentation conditions, which demonstrated significant biological activity[6]. These examples reinforce its potential not only for aroma production but also for the development of diverse high-value compounds. This thermotolerant yeast is characterized by its rapid growth, broad substrate spectrum (including lactose and inulin), high metabolic flux capacity, and resilience under stress conditions, making it attractive for industrial applications[7–10]. Its ability to metabolize L-phenylalanine (L-Phe) through the Ehrlich pathway enables the production of fusel alcohols such as 2-PE and their corresponding ester 2-PEA, particularly under nitrogen-limiting conditions[11].

In the absence of exogenous L-Phe, *K. marxianus* is capable of *de novo* synthesis of aromatic compounds via the shikimate pathway, which utilizes glucose to produce L-Phe, tyrosine, and tryptophan, subsequently feeding into the Ehrlich pathway[12]. More recently, *K. marxianus* has also been engineered to produce high-value molecules such as resveratrol by incorporating heterologous plant genes that exploit its native aromatic amino acid biosynthesis pathways[13].

While most recent biotechnological strategies rely on metabolic engineering, including overexpression of genes such as *ARO9*, *ARO10*, and *ATF1* in *Saccharomyces cerevisiae* or *Candida glycerinogenes* to boost 2-PE and 2-PEA production[14,15], native strains of *K. marxianus* such as ITD0090 can produce these compounds without genetic modification. Previous studies on engineered *K. marxianus* strains have achieved significant 2-PE production (1.3 g/L) using minimal media, demonstrating its metabolic potential[12,16].

Despite this promise, one of the main challenges associated with the production of 2-PE and 2-PEA is the cytotoxicity of these metabolites, which can impair cell growth and metabolic activity. Therefore, understanding the regulatory and metabolic mechanisms that allow certain strains to tolerate or mitigate this toxicity is critical for optimizing bioprocesses[3,9].

In this study, we explore the endogenous regulation of biosynthetic pathways in the native strain *K. marxianus* ITD0090 under nitrogen-limited conditions. Through genomic and transcriptomic analyses, we identify key metabolic features that contribute to 2-PE and 2-PEA biosynthesis without the need for genetic engineering. Our findings expand the current understanding of *K. marxianus* physiology and support its application as a sustainable platform for aroma compound production in the context of natural product biotechnology.

To our knowledge, this is the first study that integrates physiological fermentation data with genomic and transcriptomic analyses to explain the endogenous regulation of 2-PE and 2-PEA biosynthesis in a wild-type *K. marxianus* strain under nitrogen limitation. Unlike most reports focused on genetically engineered yeasts, we show that *K. marxianus* ITD0090 naturally reprograms its metabolic flux toward ester formation, favoring 2-PEA production as a potential detoxification strategy. By uncovering unique regulatory features, such as differential expression of *ARO* genes and persistence of shikimate activity in the presence of L-Phe, this work provides novel insights into alternative metabolic strategies for coping with nutrient stress. These findings not only advance the understanding of non-conventional yeast metabolism but also highlight the potential of native strains as sustainable, non-engineered platforms for aroma compound production.

Materials and methods

Biological material

A strain native to the mezcal production process in Mezquital Durango (23° 28’ 22” N, 104° 24’ 40” W), *K. marxianus* ITD0090, from the Instituto Tecnológico de Durango and belonging to the collection of the Post-harvest Technology Laboratory was used for the trials.

Fermentation with induction and nitrogen-limiting conditions: de Novo 2-PE and 2-PEA synthesis

The method described by Adame et al.[17], with some modifications, was used to carry out fermentation. Pre-inoculation of *K. marxianus* yeast ITD0090 was performed in 500-mL screw-top flasks containing 200 mL of YPD (yeast extract peptone dextrose) medium (20 g/L glucose, 20 g/L casein peptone, and 10 g/L yeast extract). The cells were incubated at 30 °C with shaking (120 rpm) for 12 h. For fermentation, the cells were recovered by centrifugation and resuspended in sterile distilled water and used to inoculate 100 mL flasks containing 50 mL of modified Etschmann’s medium[18] (30 g/L glucose, 35 g/L $Na_2HPO_4.2H_2O$, 10.5 g/L citric acid, 0.28 g/L Na_2SO_4, 0.17 g/L yeast extract, and 9 g/L L-Phe, called medium with L-Phe and without L-Phe, also called nitrogen limitation (*de novo* synthesis) at a final cell concentration of 10^7 cells/mL validated by Neubauer chamber counting and optical density. The cells were incubated at 30 °C with shaking (120 rpm) for 30 h. Experiments were carried out in triplicate, and samples were collected at 0, 8, 12, 24, and 30 h. The biomass and supernatant were frozen at −20 °C for analysis of aromatic compounds and expression analysis.

Quantification of aromatic compounds

To analyze the aromatic compounds produced by the yeast, volatile compounds were extracted using headspace solid-phase microextraction (HS-SPME) with a 50/30-mm divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS) fiber at 35 °C for 1 h. Extraction was performed with 2 mL of culture medium in 10-mL vials sealed with an aluminum cap with an olitetrafluoroethylene (PTFE)/silicone septum. Quantification was performed using gas chromatography-mass spectrometry (GC-MS) (Agilent Technologies, 6890 N) equipped with an Innowax capillary column (60 µm and 0.25 mm inner diameter, 0.25 µm film thickness). The oven temperature was maintained at 40 °C for 5 min, then increased to 220 °C in increments of 10 °C per minute, and finally maintained at 220 for 30 min. High-purity helium was used as carrier gas at 0.7 mL per minute. according to the methodology previously described by Rodriguez-Campos et al.[19]. Compounds were identified by comparison with mass spectra in the NIST database. Standard curves were used to quantify 2-PE and 2-PEA.

Genomic study and quantification of the expression of the associated pathways for 2-PE and 2-PEA production

a) **Genomic analysis**.

For the genomic exploration of the 2-PE and 2-PEA production pathways, different in silico tools were used. To search the genes, present in the shikimate and Ehrlich pathways in *K. marxianus*, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database available at https://www.genome.jp/kegg/ (L-phenylalanine, tyrosine and tryptophan biosynthesis pathway ID 00400 6/10/24 and L-phenylalanine metabolism pathway ID 00360 5/22/25) was used[20–22]. The reference genome of *K. marxianus* strain DMKU3-1042 was used to screen genes that were not found in the KEGG database and to search for transcription factors. For comparative analyses of key gene products, the amino acid sequences of *K. marxianus* strain DMKU3-1042 and *S. cerevisiae* S288C were compared using protein BLAST[23]. Primers were designed to assess the expression of key genes, and gene libraries were obtained from representative sequences reported in NCBI (available at https://www.ncbi.nlm.nih.gov/)[24]. Sequence comparison was performed by local alignments using MEGA 11 with the integrated ClustalW algorithm (available at https://www.megasoftware.net/)[25]. The genes selected for expression analysis, the primers designed, and their characteristics are listed in Table 1.

a) **Expression analysis**.

To analyze the expression of key genes, the cell pellet was recovered from each fermentation sampling time, and RNA extraction was performed using TRIsure® and liquid nitrogen. The methods established in the BIOLINETM TRIsure® kit and liquid nitrogen extraction described by Sambrock and Rusel[26] were used with some modifications. Cell lysis for the separation of protein and nucleic acids was performed with chloroform, and RNA precipitation was performed using isopropyl alcohol. RNA was washed twice with 75% ethanol. Finally, RNA was resuspended in diethylpyrocarbonate (DEPC)-treated water. The RNA concentration and quality were confirmed using Nanodrop® (ThermoFisher Scientific) and 1.5% agarose gel with 10% 3-(N-morpholino)-b propanesulfonic acid (MOPS), 10X acid buffer (3-(N-morpholino)-propanesulfonic acid), and 18% formaldehyde. To ensure DNA removal, treatment was performed with a DNase kit from Promega® according

Gene	Sequence	Tm	Amplicon (bp)
ALG9	3’-CCATCTCAGGATCCCTCTTC-5’	53.8	148
	5’-CGATTCCAGCGAATAGTTGA-3’	51.7	
UBC6	3’-CCATACGAGAATGGACAATACCACG-5’	57.7	172
	5’-CCACAGACCATGCTGGGTTCC-3’	58.3	
ARO3	3’-TCTGCGGTGTAATGATCGAGTC-5’	54.8	133
	5’-ACCTCATCAGTGGTATCCCAA-3’	52.4	
ARO4	3’-CTGGTTTATCCTTCCCAGTCG-5’	54.4	191
	5’-TGTTCCCTTCTTACCACCTC-3’	51.8	
PHA2	3’-ATTGGAAGTCGTGCTAGTGC-5’	58.4	120
	5’-GCGATTATTCGGCTTACGCCTTC-3’	57.1	
ARO8	3’-AATTCGGACAACCACAGAGCA-5’	52.4	186
	5’-GCCCTATAACCCAGCCTAATC-3’	54.4	
ARO9	3’-GACGGTACGATCACCTATGAC-5’	54.4	63
	5’-AATTGTGAGGTAGGATGGCGT-3’	52.4	
ARO10	3’-GATCTTGTGTGAAGGTGATGGTG-5’	55.3	141
	5’-CTGGTTGGTCCTAGAATAGC-3’	51.8	
ARO80	3’-GCTCTCTGTACTATCACCGTTG-5’	54.8	294
	5’-TTCGCAGCGTTGTATGCAAG-3’	51.8	
CAT8	3’-CGCTCGTGGTTCGTTAGAGTAC-5’	56.7	132
	5’-CTATGCCAGCTCATTGTGC-3’	57.5	
MIG1	3’-GCTCAAACATGGGATTCGGAACC-5’	57.1	120
	5’-CCTTCTCATCACCAGCACTACG-3’	56.7	

Table 1. Primers designed for this study.

to protocol specifications. cDNA was synthesized following the protocols recommended by QIAGEN for the Omniscript® reverse transcription kit. Endpoint PCR was used to standardize the amplification conditions of the designed primers (Table 1), including annealing temperature and cycle parameters.

RT-qPCR was performed following the methodology established by BIO-RAD for the Sso Advanced™ Universal SYBR® Green Supermix Kit. Finally, data analyses were performed according to the specific instructions for the CFX96 Thermocycler™. All assays were performed in biological duplicate and experimental triplicate. Gene expression data were analyzed and normalized using the $\Delta\Delta C_t$ method[27]. After evaluating the expression stability of multiple candidate reference genes, *ALG9* and *UBC6* were selected as the most suitable for normalization under the tested conditions[28].

The equation used in the ΔΔCt method for the relative quantification of gene expression was as follows:

$$\Delta\ \mathrm{C}_t = \mathrm{C}_t\ \text{target gene} - \mathrm{C}^t\ \text{reference gene.}$$

$$\Delta\Delta\ \mathrm{C}_t = \Delta\ \mathrm{C}_t\ \text{sample} - \Delta\ \mathrm{C}_t\ \text{control.}$$

$$\text{Ratio of expression} = 2 - \Delta\Delta\ \mathrm{C}t.$$

where C_t is the amplification threshold cycle; ΔC_t corrects for differences in initial RNA loading; $\Delta\Delta C_t$ compares the relative expression between the experimental sample and the control conditions; and $2^{-\Delta\Delta Ct}$ determines the relative expression of the gene of interest.

Statistical analysis

The data for the aromatic compounds 2-PE and 2-PEA were assessed using the HSD-Tukey-Kramer comparison test at $\alpha = 0.01$. All statistical analyses were performed using JMP software version 13.2 (SAS Institute, Inc., NC, USA).

Results and discussion

Aromatic compound production

Aromatic compounds produced by the yeast *K. marxianus* were detected after cultivation under nitrogen-limiting conditions (*de novo* synthesis) and induction conditions for the activation of these compounds. Those produced by the Ehrlich route from phenylalanine were identified. The complete aromatic composition detected in our fermentation assays, including alcohols, esters, and fatty acids with relevant organoleptic properties, is summarized in Table 2. These metabolites represent the compounds of greatest interest for fermentation-derived aroma production. The presence of esters derived from higher alcohols and fatty acids was particularly notable, as these metabolites contribute fruity and floral notes to fermented products. Similar profiles of volatile esters have been reported in previous studies[17,29], where they were associated with the distinctive aroma of alcoholic beverages produced from musts[30–32]. The presence of phenylalanine in the culture medium favors aroma production. It has been reported that isoamyl acetate, 2-PE, and 2-PEA are produced *de novo* independent of a nitrogen source[29]. In addition, fermentations of agave must with *K. marxianus* under nitrogen-limiting conditions have been reported to preferentially enhance the production of 2-PE and 2-PEA[33]. This suggests that the nitrogen composition of the natural substrate plays a key role in redirecting metabolic flux toward the Ehrlich pathway, thereby favoring the accumulation of aromatic alcohols and esters during long fermentation processes. The production of acetate esters, such as isoamyl acetate and 2-PEA, is carried out by synthesizing acetyl-CoA and alcohol (isoamyl and 2-PE, respectively) and an alcohol acetyltransferase enzyme (AATase). This synthesis is regulated by parameters such as the presence of unsaturated fatty acids, precursor molecules, nitrogen sources, and oxygen. However, the most determining factors are the availability of the substrate (higher alcohol), the levels of acetyl-CoA in the cytoplasm, and the expression and activity of alcohol acetyltransferase enzymes[32,34–36].

The metabolites that could be quantified in this study corresponded to 2-PE and 2-PEA, the final metabolites of the Ehrlich pathway produced by the presence of L-Phe as an inducer. Production occurred both in *de novo* synthesis via the shikimate pathway and subsequent incorporation into the Ehrlich pathway to produce these aromatic metabolites[12]. Additionally, 0.227 g/L of 2-PE and 0.630 g/L of 2-PEA were produced by *de novo* synthesis. When production was induced with L-Phe, 0.435 g/L of 2-PE and 1.0 g/L of 2-PEA were produced (Table 3 **and Table S1**). Studies have shown that 2-PE and 2-PEA production in *K. marxianus* strains varies depending on the composition of the culture medium and the strain studied. Ivanov et al.[37] used *K. marxianus*

Compound	RT	Formula	m/z	Sensorial description
Isoamyl acetate	6.8	$C_7H_{14}O_2$	87-70-61-55-43	Banana, fruity and sweet
Isoamyl alcohol	9.5	$C_5H_{12}O$	70-55-41	Banana, fruity and malt
Benzaldehyde	19.8	C_7H_6O	84-77-63-51	Almonds, cherry and sweet
2-Phenylethyl acetate	28.8	$C_{10}H_{12}O_2$	104-91-78-65-51-43	Floral, fruity and sweet
2-Phenylethyl propanoate	30.5	$C_{11}H_{14}O_2$	104-91-77-57-41	Floral, rose and sweet
2-Phenylethanol	31.3	$C_8H_{10}O$	103-91-77-65-51	Roses, fresh and leafy

Table 2. Volatile metabolites synthesized by *K. marxianus* ITD0090 during fermentation under nitrogen limitation and L-Phe supplementation.

Production of 2-PEA (g/L)							
Time	Whit L-Phe inductor			Nitrogen limitation			
(h)	Mean		S.E.	Mean		S.E.	P-*value*
8	0.170	±	0.038	0.133	±	0.001	0.4352
30	1.00	±	0.035	0.630	±	0.030	0.0153*
Production of 2-PE (g/L)							
Time	**Whit L-Phe inductor**			**Nitrogen limitation**			
(h)	**Mean**		**S.E.**	**Mean**		**S.E.**	**P-*value***
8	0.223	±	0.023	0.149	±	0.011	0.0994
30	0.435	±	0.020	0.270	±	0.034	0.0334*

Table 3. Comparison of 2-PEA and 2-PE production by *K. marxianus* ITD0090 under L-phenylalanine-induced and nitrogen-limited (non-induced) conditions. *Significantly different means in the comparison of the effect in the presence/absence of L-phenylalanine.

NBIMCC1984 and *K. marxianus* 35 to evaluate aroma production in culture with L-Phe, achieving 0.88 and 1.10 g/L of 2-PE after 36 h of culture. Wittmann et al.[38] evaluated *K. marxianus* strain CBS 5670 for 2-PE and 2-PEA production with carbon sources, achieving a maximum production of 15 mM 2-PE at 40 h and 5 mM 2-PEA at 15 h. However, 2-PEA production stopped at 22 h. In contrast, in our results, production had not ceased after 30 h, indicating that bioconversion to 2-PE and 2-PEA continued to be active, with no decrease in biomass production or cell viability. The activity of the Ehrlich pathway is associated with growth, particularly in the exponential phase, so if cells continue to grow, both metabolites continue to be produced[39]. Increased production occurs once nitrogen and carbon sources are consumed, although intoxication by end products, such as ethanol and 2-PE, can inhibit *K. marxianus* metabolism[32,40,41], in addition to feedback blocking the production pathway[42]. However, in this study, neither growth inhibition nor intoxication phenomenon occurred, as metabolism was more efficient for ester production. Garavaglia et al.[43] determined that without L-Phe as a precursor and optimization of the culture medium, 2-PE production by *K. marxianus* was usually low, generally between 0.01 and 0.10 g/L. The fermentation model, with a limited nitrogen source of 0.17 g/L yeast extract and 9 g/L L-Phe[18], resembles the natural conditions of agave must, in which *K. marxianus* ITD0090 can develop long fermentation times without compromising biomass production, producing a variety of aromatic compounds[17,33,44]. Tolerance to toxic products is crucial for optimizing production in bioprocesses, and strategies such as in situ product removal (ISPR) and adaptation and selection of tolerant strains have been shown to significantly improve productivity. ISPR methods, such as adsorption on resins and extraction in two-phase systems, mitigate product inhibition and increase the fermentation efficiency[45–48]. In addition, through the hybrid combination of gas extraction and vacuum evaporation, recovery processes have been shown to be effective in the initial separation of these products from liquid culture[27].

Genetic engineering and synthetic biology have driven advances in the microbial production of 2-PE and 2-PEA. Several microorganisms have been modified to optimize metabolic pathways and improve their efficiency. Various strategies have enhanced 2-PE production in microorganisms: *C. glycerinogenes* reached 5.0 g/L via overexpression of *AAP9*, *ARO9*, *ARO10*, and *SLC1*[49]; *S. cerevisiae*, 4.02 g/L through pathway fusion and *PDC5* deletion[50]; a consortium of *E. coli* and *M. guilliermondii*, 3.77 g/L[51]; while *K. marxianus* produced >0.800 g/L using minimal media[12].

2-PEA production has also been enhanced in bacterial systems. In *E. coli*, overexpression of *aroG fbr*, *pheA fbr*, *atf1*, and *aro8*, along with heterologous *KDC* and *yjgB*, yielded 1 g/L 2-PE and 0.687 g/L 2-PEA[52]. In *Pseudomonas putida* DOT-T1E, plasmid-based expression of Ehrlich pathway genes boosted 2-PEA production to 100 ppm from glucose[53].

In this study, competitive production was achieved without genetic modification, indicating that *K. marxianus* strain ITD0090 can be considered a potential microbial chassis for the biosynthesis of aromatic compounds, offering a favorable cost-benefit ratio by not requiring supplementation with expensive amino acids or nitrogen sources based on ammonium salts that limit aroma production due to transcriptional inhibition of metabolic pathways.

Genomic study and quantification of the expression of the associated pathways for 2-PE and 2-PEA production

a) **Via the shikimate pathway**.

2-PE and 2-PEA can be produced *de novo* through the shikimate pathway, which uses phosphoenolpyruvate (PEP), a derivative of glycolysis, and erythrose-4-phosphate (E4P), a product of the pentose phosphate pathway, as substrates. In yeast, the 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthases Aro3 and Aro4 initiate the biosynthesis of L-phenylalanine, tyrosine, and tryptophan, converting E4P and PEP to DAHP. These enzymes are regulated through a negative feedback process in which Aro3 is inhibited by L-Phe and high concentrations of tyrosine and tryptophan and Aro4 are inhibited by the presence of tyrosine and high concentrations of L-Phe and tryptophan[54]. The *K. marxianus* reference strain possesses a single copy of the *ARO3* and *ARO4* genes. However, analysis of its conserved domain revealed structural differences compared to *S. cerevisiae* (Table 4), suggesting possible variations in their function. In expression analyses, *ARO3* was

Gene	K. marxianus			S. cerevisiae		
	Locus tag	**Aa**	**Conserved Domain**	**Locus tag**	**Aa**	**Conserved Domain**
ARO3	KLMA_40107	372	AroG1	YDR035W	370	AroFGH
ARO4	KLMA_20414	364	AroG1	YBR249C	370	AroFGH
PHA2	KLMA_70162	311	PBP2_PDT_like, ACT_CM-PDT.	YNL316C	334	PBP2_PDT_like, ACT_CM-PDT
ARO8 1	KLMA_20259	497	Aro8	YGL202W	500	Aro8
ARO8 2	KLMA_50151	477	Aro8	Not found in *S.c.*		
ARO8 3	KLMA_20431	451	Aro8	Not found in *S.c.*		
ARO9	KLMA_60423	530	Aro8	YHR137W	513	Aro8
ARO10	KLMA_20597	630	PDC1	YDR380W	635	PDC1
PDC1	KLMA_60075	564	PDC1	YLR044C	563	PDC1
PDC1 2	KLMA_40053	578	PDC1	Not found in *S.c.*		
ADH	KLMA_40624	386	FDH like ADH2	Not found in *S.c*		
ADH1	KLMA_40102	348	CAD3	YOL086C	348	CAD3
ADH2	KLMA_40220	348	CAD3	YMR303C	348	CAD3
ADH3	KLMA_80306	351	CAD3	YMR083W	375	CAD3
ADH4A	KLMA_20005	418	PDDH	YGL256W	382	PDDH
ADH4B	KLMA_20158	379	CAD3	Not found in *S.c*		
ADH5	Not found in *K.m.*			YBR145W	351	CAD3
ADH6	KLMA_80339	366	CAD1	YMR318C	360	CAD1
ADH7	Not found in *K.m.*			YCR105W	361	CAD1
SFA1	KLMA_50315	384	Alcohol DH III	YDL168W	386	Alcohol DH III
ALDA	KLMA_20672	583	ALDH-SF	Not found in *S.c*		
ALD2	KLMA_20673	581	ALDH-SF	YMR170C	506	ALDH_ALD2
ALD22A	KLMA_30421	640	ALDH_F15-22	Not found in *S.c*		
ALD3	Not found in *K.m.*			YMR169C	506	ALDH-SF
ALD4	KLMA_50012	521	ALDH_F1-2_Ald2-like	YOR374W	519	ALDH_F1-2_Ald2-like
ALD5	KLMA_40404	514	ALDH_F1-2_Ald2-like	YER073W	520	ALDH_F1-2_Ald2-like
ALD6	KLMA_10742	507	ALDH_F1-2_Ald2-like	YPL061W	500	ALDH_F1-2_Ald2-like
ATF1	KLMA_30203	515	AAtase	YOR377W	525	AATase
ATF2	Not found in *K.m.*			YGR177C	535	AATase
ARO80	KLMA_30668	924	GAL4	YDR421W	950	GAL4, Fungal_TF_MHR
CAT8	KLMA_80046	1412	Fungal_trans, GAL4, ZIP-Cat8	YMR280C	1433	Fungal_trans, GAL4, ZIP-Cat8
MIG1	KLMA_20059	556	COG5048	YGL035C	504	COG5048
GLN3	KLMA_30682	797	ZnF-GATA, GAT1	YER040W	730	GAT1
GAT1	Not found in *K.m.*			YFL021W	510	GAT1

Table 4. Comparative analysis of the amino acid sequence of the evaluated genes. The gene identifier, amino acid length, and predicted conserved domain are included. * The reference strains *K. marxianus* DMKU3-1042 and *S. cerevisiae* S288C were used.

overexpressed at 8 h, maintaining this level up to 12 h under both experimental conditions. *ARO4* showed overexpression at 8 h in the presence of L-Phe, and its expression doubled during *de novo* synthesis, indicating a possible differential regulatory mechanism for this strain.

The presence of aromatic amino acids (AAAs) has been reported to repress Aro3 due to its affinity for L-Phe and the negative feedback mechanism. Both genes are regulated by the general control system of amino acid biosynthesis, mediated by the activator Gcn4[55–57]. This system allows the derepression of genes under amino acid shortage, suggesting the possible intervention of other factors that favor their expression, as shown in **Figure S1**. This dysregulation could be due to the tyrosine-insensitive alleles of *ARO4*, which significantly increase the intracellular concentration of phenylalanine and tyrosine, promoting the production of derived aromatic compounds[58,59]. Interestingly, the overexpression of *ARO3* and *ARO4*, as key players in the *de novo* synthesis of phenylalanine, is consistent with the observations of[60], who posited that the overexpression of genes related to the Ehrlich pathway, including *ARO9* and *ARO10*, could increase 2-PE and 2-PEA production.

Dihydroxyacetone phosphate (DHAP) is converted to chorismate through the action of the Aro1 and Aro2 enzymes. From this point, chorismate is channeled into the production of prefenate, which is subsequently transformed into phenylpyruvate. At this stage, the synthesis of the amino acids L-Phe and tyrosine is regulated by Aro7, and the conversion of chorismate to anthranilate, a precursor of tryptophan, is mediated by Trp2 and Trp3. The *PHA2* gene plays a key role in the distribution of shikimate pathway intermediates to phenylalanine synthesis. Gene expression analyses showed that *PHA2* was active in presence and absence of L-phenylalanine as inducer, with expression levels between 1.7 and 3 times higher than the threshold during fermentation. These results confirm the *de novo* synthesis of phenylalanine from precursors of the shikimate pathway. However, previous studies have reported that Pha2 activity may be regulated by the intracellular L-Phe concentration[12,61]. High phenylalanine concentrations have been observed to inhibit Pha2 enzymatic activity, limiting its function and preventing its excessive overproduction. However, in *K. marxianus* ITD0090, this negative feedback mechanism was not detected, suggesting differential regulation of the pathway in this strain. Once L-Phe is produced, it is used to produce 2-PE and 2-PEA via the Ehrlich pathway. This route has been used as an inducer system by exogenously adding L-Phe and stimulating the production of these metabolites.

a) Ehrlich pathway

In *S. cerevisiae*, the first step of transamination of L-Phe to phenylpyruvate is catalyzed by amino acid transaminase isoenzymes associated with the *ARO8* and *ARO9* genes. In the results obtained from the in silico analysis performed using the Ehrlich pathway in *K. marxianus* (Table 4), this first step can be catalyzed by four genes encoding three copies of the *ARO8* gene and one copy of the *ARO9* gene. This represents an overrepresentation of genes in this step of the pathway with respect to *S. cerevisiae*. Both Lertwattanasakul et al.[62] and Rajkumar and Morrissey[12] discussed the presence of one copy of the *ARO8* gene; however, three duplicated proteins were found to share features, such as the same functional aminotransferase domain, lysine residue as the catalytic site, enzyme folding sites, and pyridoxal 5'-phosphate binding and homodimerization sites, suggesting their functional potential.

According to in silico analysis, *ARO9* is conserved in a single copy, as in *S. cerevisiae*. The first step can also be catalyzed by other aminotransferase enzymes with similar functional domains, such as *AAT1*, *AAT2*, and *HIS5*, reported with a single copy in the genome of *K. marxianus*. However, these enzymes have been related more to the synthesis of aspartate, asparagine, and histidine[63].

ARO8 expression levels remain below threshold and are significantly lower in the presence of L-Phe (Fig. 1). This repression of aminotransferase expression contradicts literature reports. Although Romagnoli et al.[61] reported that *ARO8* is constitutively expressed and participates in L-Phe degradation with particularly low expression levels, the observed values were almost 0, despite having 3 copies of the gene. In contrast, in the absence of a nitrogen source, *de novo* synthesis resulted in an expression level close to the threshold representing constitutive expression, with a downward trend towards 30 h, which could be considered a repression in the expression of this gene. Studies have suggested that *ARO8* repression in yeast is influenced by catabolic nitrogen and glucose repression, affecting 2-PE production and the expression of related genes[14,64].

However, *ARO9* showed overexpression in the presence of L-Phe, with two major peaks: the first at 8 h with an 8-fold overexpression and the second at 24 h with a 22-fold overexpression (**Figure S1**). Romagnoli et al.[60] reported that *ARO9* was inducible by aromatic aminoacids, such as L-Phe, tyrosine, and tryptophan, participating in the degradation of the latter when in excess. In *de novo* synthesis, *ARO9* overexpression was even higher, maintaining this overexpression throughout fermentation.

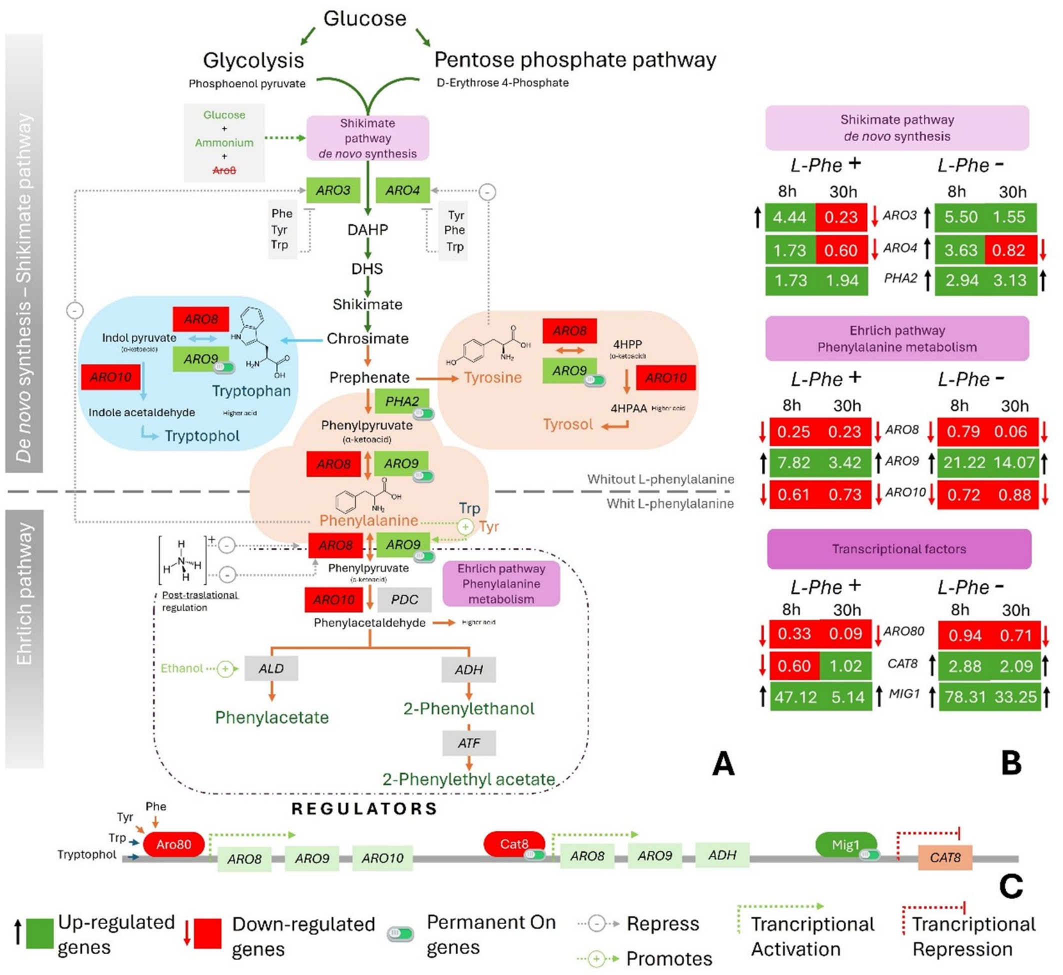
The apparent inactivity of *ARO8* in *K. marxianus* strain ITD0090 contrasts with reported results[14,61]. Because there is variation in the expression levels, at least one of the copies has activity, in addition to the presence of a functional analogue, such as *ARO9*, which could be the main analogue. Although *ARO9* showed different kinetic properties from *ARO8*, elements outside the gene architecture, such as position in the genome, regulatory modules, transcriptional factors, and even null mutations in *ARO8* in any of the copies, can be partially complemented by it, which may have occurred in this study.

The apparent *ARO8* dysfunction is notable, as mutations in essential metabolic genes are uncommon. This may reflect adaptive evolution in *K. marxianus*, a yeast known for its superior fermentation capacity and metabolic efficiency compared to *S. cerevisiae*, largely due to its distinct genome structure[62]. Transcriptomic analyses reveal specialized pathways for oxidative stress tolerance, rapid growth, and high metabolite yields, supported by an overrepresented transporter system that enhances enzyme mobilization and overall metabolic performance[7,8].

Aro8 has been reported to not be functional when the main nitrogen source is ammonium. However, in this study, the main nitrogen source was L-Phe. Wang et al.[14] showed that ammonium sulfate did not affect *ARO8* expression but significantly decreased enzymatic activity, which was related to lower 2-PE production. Mares-Rodriguez et al.[44] suggested a constant production of ammonium by environmental nitrogen fixation due to the presence of the endosymbiont bacterium, *Bacillus tequilensis*, but no decrease in 2-PE production was observed, which could be attributed to the fact that *ARO9* has this function.

Deletion of *ARO8* has been reported to trigger *de novo* production of L-Phe, which increases 2-PE production in cultures using glucose and ammonium as nitrogen sources[12,61]. Although our experimental conditions were not identical, we observed that the level of *ARO8* transcripts in both conditions was very low. *ARO9* overexpression in the absence of the inducer is evidence of *de novo* L-Phe synthesis. In the presence of the inducer, *de novo*-produced L-Phe was targeted for conversion to 2-PE and subsequently 2-PEA, along with L-Phe present in the medium, resulting in high concentrations of these aromatic compounds. *ARO9* overexpression is a subject of interest in metabolic engineering for 2-PE and 2-PEA production, as *ARO9* and *ARO10* overexpression together has been shown to significantly increase 2-PE production[60]. However, *K. marxianus* ITD0090 appears to only require *ARO9* for that purpose.

The second step of the Ehrlich pathway involves the decarboxylation of phenylpyruvate to synthesize phenylacetaldehyde. In *S. cerevisiae*, this reaction is catalyzed by three pyruvate decarboxylase isoenzymes, Pdc1, Pdc5, and Pdc6, in addition to the phenylpyruvate decarboxylase Aro10, which generally has a higher affinity for the substrate[11,38,65]. In the genome annotated by the KEGG database of *K. marxianus*, Aro10 was the only enzyme predicted in this reaction. However, two homologous genes containing the pyruvate decarboxylase



Shikimate pathway de novo synthesis

	L-Phe +		L-Phe −	
	8h	30h	8h	30h
ARO3	↑ 4.44	0.23 ↓	↑ 5.50	1.55
ARO4	1.73	0.60 ↓	↑ 3.63	0.82 ↓
PHA2	1.73	1.94	↑ 2.94	3.13 ↑

Ehrlich pathway Phenylalanine metabolism

	L-Phe +		L-Phe −	
	8h	30h	8h	30h
ARO8	↓ 0.25	0.23 ↓	↓ 0.79	0.06 ↓
ARO9	↑ 7.82	3.42 ↑	↑ 21.22	14.07 ↑
ARO10	↓ 0.61	0.73 ↓	↓ 0.72	0.88 ↓

Transcriptional factors

	L-Phe +		L-Phe −	
	8h	30h	8h	30h
ARO80	↓ 0.33	0.09 ↓	0.94	0.71 ↓
CAT8	↓ 0.60	1.02	↑ 2.88	2.09 ↑
MIG1	↑ 47.12	5.14 ↑	↑ 78.31	33.25 ↑

Fig. 1. **Comparison of metabolic pathways and gene expression levels of*K. marxianus*ITD0090 with and without L-phenylalanine as inducer. (A)** Shows the deregulation of the Shikimate and Ehrlich pathways in the presence and absence of L-phenylalanine, highlighting the differences in key gene expression and metabolic fluxes. Metabolic pathway based on that reported in KEGG[20–23]. **(B)** Shows that *ARO8*, *ARO9*, *ARO10* and other key factors are differentially regulated (green: overexpression, red: repression). Numerical values represent relative expression levels under each condition, while **(C)** Illustrates the genes that are transcription factors and the genes they regulate. Arrows indicate transcription or repression action.

(PDC) domain were identified in the *K. marxianus* genome (Table 4). Moreover, Rajkumar and Morrissey[12] identified a *PDC5* gene, whose enzymatic activity is not yet fully characterized, but which could also function as a possible analog for this step. In this study, *ARO10* showed low expression levels in *de novo* synthesis and induction with L-Phe, which remained stable throughout fermentation under both conditions. This result was not related to the kinetics of 2-PE or 2-PEA production, which were produced after 8 h of fermentation (**Table S1**).

Romagnoli et al.[66] noted that only Aro10 and Pdc5 could decarboxylate phenylpyruvate, whereas Pdc1 and Pdc6 did not exhibit this activity, suggesting that low Aro10 levels could be compensated for by the action of another enzyme with pyruvate decarboxylase activity. Furthermore, in silico analyses revealed that the conserved domain of *ARO10* in *K. marxianus* was similar to that of the two copies of *PDC1*, indicating a functional analogy between these enzymes and reinforcing the idea of compensatory activity. However, the slight difference in *ARO10* expression levels at 30 h could be related to high cell density, which coincides with the time of highest aroma production during the stationary phase according to Wang et al.[67].

Once phenylacetaldehyde is formed, it can be reduced to form 2-PE or oxidized to form phenylacetate. To produce phenylacetate (PA), which competes with 2-PE, we identified six aldehyde dehydrogenase enzymes, which have also been reported to be present in *S. cerevisiae*, with some located in the cytosol (Ald2, Ald3, and Ald6) and others in the mitochondria (Ald4, Ald5, and Hfd1). However, only Ald4 and Ald6 have been shown to participate in acetate formation.

In *S. cerevisiae*, the reduction of phenylacetaldehyde to form 2-PE is performed by several alcohol dehydrogenases (Adh1, Adh2, Adh3, Adh4, and Adh5) or formaldehyde dehydrogenase Sfa1[68]. In *S. cerevisiae*, this step, which is not yet fully elucidated in KEGG, has not identified the specific gene that acts as an alcohol dehydrogenase in L-Phe metabolism in *K. marxianus*. In our reference genome analysis, eight genes were detected with this possible function (Table 4). Some yeasts, such as *Starmerella bacillaris*, have confirmed that the enzyme Adh5 is responsible for 2-PE production[69], and in *K. marxianus*, several isoenzymes of alcohol dehydrogenase (Adh) were identified and characterized, of which four genes stand out: *KmADH1*, *KmADH2*, *KmADH3*, and *KmADH4*. In general, the *ADH* genes show high sequence similarity and differential expression according to growth phase and carbon source, suggesting that each have specialized functions within the cell[70].

2-PE is transformed into 2-PEA by transesterification catalyzed by alcohol acetyltransferase (Aat) enzymes, using acetyl-CoA and 2-PE as the main substrates in a process associated with their degradation[68,71–73]. AATs are bisubstrate enzymes that catalyze the transfer of acyl groups from an acyl-CoA donor to an acceptor alcohol[74,75]. In *S. cerevisiae*, the enzymes Atf1p and Atf2p are responsible for the synthesis of various acetate esters, whereas in *K. marxianus*, only *ATF1* has been identified, which retains the functional domain of its homologue in *S. cerevisiae*[76,77]. The overexpression of *ATF1P* and *ATF2P* in *S. cerevisiae* has been reported to significantly increase acetate ester production[78–80]. In addition, there is evidence that Atf2p plays a key role in sterol detoxification[76]. AATs are not only involved in ester synthesis but also play essential roles in the detoxification of ethanol and other metabolites, CoA recycling from acyl-CoA, and fatty acid metabolism[76,77,81,82]. These findings suggest that ester production is not solely dependent on TAA activity but is closely linked to metabolic mechanisms that protect cellular integrity against the dysfunction of pathways essential for survival[83].

A 9:1 ratio of 2-PE to 2-PEA is produced in *S. cerevisiae*[84,85]. In contrast, we obtained a 1:2 ratio between 2-PE and 2-PEA. These differences can be attributed to the addition of L-Phe[3]. Some strategies have been implemented to increase 2-PEA production, such as co-fermentation. The combination of *K. marxianus* and *M. guilliermondii* can limit 2-PE accumulation and favor conversion to 2-PEA[86]. Similarly, co-fermentation of *Hanseniaspora vineae* and *S. cerevisiae* showed a synergistic effect that significantly increased 2-PEA production possibly due to the metabolic complementarity between the species[87]. The gene pool of each strain also influences 2-PEA production; differences in enzymatic activity and gene regulation have been reported as determining factors. Variants of the *ARO80* gene in different *Saccharomyces* species modify the conversion of L-Phe to 2-PEA[83].

The genetic basis of aromatic compound synthesis in yeasts is complex and species-specific[88]. While the shikimate pathway contributes, its efficiency is limited as sugars are prioritized for growth[2]. In contrast, *K. marxianus* favors volatile production via L-Phe biotransformation through the Ehrlich pathway[89,90].

Transcription factors involved in 2-PE and 2-PEA production

The regulation of the Ehrlich pathway, which is central to the biosynthesis of 2-PE and 2-PEA, is a complex and multifactorial process influenced by transcriptional regulation, nutrient availability, and environmental conditions. The transcription factor Aro80 has been identified as a key activator of *ARO9* and *ARO10* expression in response to aromatic amino acids such as L-phenylalanine[85,91]. However, its activity is modulated by additional elements, including GATA transcription factors (Gln3p, Gat1p, and Nil1p), which are responsive to nitrogen availability[9].

Further complexity is added by regulators such as Gcn4p and Gln3p, which respond to amino acid availability, and carbon metabolism regulators including Mig1p, Rgt1p, and Nrg1p, which mediate glucose repression[92,93]. Factors involved in stress and nutrient limitation, such as Hsf1p (heat stress), Cat8p, Sip4p, Adr1p (glucose starvation), and Tye7p, Gcr1p, Ert1p, and Tda9p (growth and adaptation), may also contribute to the modulation of this pathway (Figure S2)[94–96].

In silico analyses revealed differential expression of *MIG1* and *CAT8* during fermentation, suggesting that multiple overlapping transcriptional programs co-regulate aromatic compound production. These findings support the idea that 2-PE and 2-PEA biosynthesis is governed by a dynamic and interconnected regulatory network sensitive to nitrogen and carbon status.

Impact of key gene deregulation of the Shikimate and Ehrlich pathways on 2-PE and 2-PEA production

Our analyses revealed that deregulation of genes, such as *ARO8* and *ARO9*, significantly influenced 2-PE and 2-PEA production. The absence of *ARO8* at the transcriptomic level in both experimental conditions seemed to favor *de novo* phenylalanine synthesis, which increased 2-PE production, especially in cultures with glucose and ammonium as carbon and nitrogen sources[12,61]. In the presence of L-Phe as an inducer, the high activity of Aro9, with a higher affinity for tryptophan (K_m 0.4 mM) than for phenylalanine (K_m 0.2 mM) or tyrosine (K_m 0.2 mM), prioritized tryptophan degradation, releasing feedback inhibition of *ARO3* and *ARO4*. This activated the shikimate pathway, producing first tryptophan and then L-Phe and tyrosine, which subsequently inhibited *ARO3* and *ARO4* in the advanced stages of fermentation. In the absence of L-Phe, *de novo* AAA synthesis was directly activated, with Aro3 and Aro4 operating until 12 h, at which time tryptophan, L-Phe, and tyrosine production inhibited these enzymes. Aro80 expression, induced by tryptofol (tryptophan metabolite), and sustained Pha2 activity confirmed *de novo* L-Phe production in the presence and absence of L-phenylalanine as inducer.

The production of 2-PE and 2-PEA in the presence of L-Phe was derived from both the added inducer and *de novo* synthesis, whereas in its absence, it depended exclusively on the latter. *ARO9* overexpression compensated for the inhibition of *ARO8*, driving both pathways. However, the low activity of *ARO10* and *ARO80* could be related to their repression in the presence of preferred nitrogen sources, such as glutamine and ammonium[60]. These findings highlight the regulatory complexity of the shikimate and Ehrlich pathways, suggesting the involvement of other yet unidentified genes in the production of amino acids and their derived metabolites. The phenotypic variability observed in yeast underscores the need for further studies to elucidate these mechanisms.

Our results indicate that the 2-PEA production pathway could be favored at the enzymatic level due to similarities in gene architecture and regulation, which could have significant implications for yeast physiology. In addition, 2-PEA production could be a cell strategy for detoxification. Although some enzymes are constitutively expressed, others are induced under stress or high alcohol concentrations, and function to detoxify harmful chemical intermediates and break down toxic aldehydes accumulate under stress conditions. These enzymes are usually repressed by glucose; thus, high 2-PEA production could be a defense mechanism for the cell to handle high 2-PE concentrations in the medium[97–99].

Conclusions

Under nitrogen-limited conditions, *K. marxianus* ITD0090 consistently favored 2-phenylethyl acetate (2-PEA) over 2-phenylethanol (2-PE). With L-phenylalanine (L-Phe) supplementation, titers reached 1.00 g/L 2-PEA and 0.435 g/L 2-PE; under de novo conditions, 0.630 g/L 2-PEA and 0.227 g/L 2-PE were obtained within 30 h. Transcriptome analyzes at 8 h and 30 h indicated upregulation of Ehrlich-pathway genes, notably *ARO9*, while *ARO8* showed low expression, suggesting possible functional compensation by *ARO9*. Shikimate-pathway genes (*ARO3*, *ARO4*) were expressed early and declined by 30 h, more evidently without L-Phe, consistent with a transient de novo contribution to phenylalanine supply. Together, these observations support a nitrogen-responsive shift that favors ester formation and are consistent with a hypothesis in which conversion of 2-PE to 2-PEA may mitigate product toxicity. No overt growth inhibition was observed during the 30 h window. Nevertheless, this study has limitations, as it was based on a single wild isolate and gene-expression profiling without direct flux or enzyme activity measurements. Future work should benchmark these findings against reference strains, explore targeted gene perturbations (*ARO8* complementation or AATase regulation), and examine the influence of nitrogen and carbon sources, acetyl-CoA availability, and transcriptional regulators such as Aro80, GATA factors, and Mig1p-Cat8p. These directions will help establish causality, optimize titers, and guide the sustainable application of *K. marxianus* as a microbial platform for aroma compound production.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Conceptualization: KJGL, OMRQ and ETAC; investigation and data curation: KJGL, SNRF, OMRQ and ETAC; methodology: KJGL, SNRF, SMCR, JRC, OMRQ and ETAC; validation, formal analysis, and resources: KJGL, SNRF, SMCR, JRC and ETAC; writing-original draft preparation: KJGL and ETAC; writing-review and editing: KJGL, SMCR, JRC, OMRQ and ETAC; supervision: KJGL, SMCR, JRC and ETAC; project administration and funding acquisition: ETAC.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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