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National University of Ireland, Cork

&

L'institut Agro, Montpellier, France

Contribution of the interaction between environment and genotype to flavour and aroma profile in non-traditional yeast

Thesis presented by

Angela Coral Medina, BSc, MSc

orcid.org/0000-0002-0466-9436

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University College Cork

School of Microbiology

Head of School: Prof. Paul O'Toole

Supervisor: Prof. John Morrissey

L'Institute Agro Montpellier

Sciences Pour l'Oenologie, INRAE

Head of Unit: Prof. Fabienne Remize

Supervisor: Dr. Carole Camarasa

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Contribution des interactions entre l'environnement et le génotype à la production d'arômes par les levures non-traditionnelles

Présentée par Angela CORAL-MEDINA
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Sous la direction de Prof. John MORRISSEY
et Dr. Carole CAMARASA

Devant le jury composé de

José Manuel GUILLAMÓN, Professeur, CSIC, Valencia, Spain	Rapporteur
Isabelle MASNEUF-POMAREDE, Professeure, Bordeaux Sciences Agro, France	Rapportrice
Elke ARENDT, Professeure, UCC, Cork, Ireland	Examinatrice
Fabienne REMIZE, Professeure, UM, Montpellier, France	Examinatrice
John MORRISSEY, Professor, UCC, Cork, Ireland	Directeur de thèse
Carole CAMARASA, Directrice de Recherche, INRAE, Montpellier, France	Directrice de thèse

UNIVERSITÉ DE MONTPELLIER

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University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

L'INSTITUT agro Montpellier

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Declaration

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Publications from this thesis

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Chapter 2: Transcriptional landscape of *Saccharomyces uvarum* when growing using preferred and non-preferred nitrogen sources. To be submitted.

Chapter 3: The evolution and role of the periplasmic asparaginase Asp3 in yeast. Submitted to *FEMS Yeast Research.* Minor revision.

Chapter 4: The use of alternative Transcription Start Sites controls the production of cytoplasmic or mitochondrial forms of branched-chain aminotransferase in *Kluyveromyces marxianus*. Submitted to *Genetics.* Major revision.

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Abstract

Yeast is used for fermentation in the biotechnology sector and plays a critical role in the production of fermented beverages. The quality of the final product depends on yeast performance and the environmental conditions of fermentation. Combining knowledge on yeast biology and the different fermentation elements allows generation of beverages with new properties and improved processes. Nitrogen is an essential nutrient for yeast, nonetheless, besides the contribution of the nitrogen source to growth, some nitrogen compounds are precursors of volatile molecules that produce aroma. The nitrogen compounds assimilated by yeast are classified as rich or poor nitrogen sources depending on how they support growth. Although *Saccharomyces cerevisiae* is the most important and most widely used yeast species, other non-traditional yeasts are attracting industrial interest because of the new traits of technological interest that they offer. *Saccharomyces uvarum* has relevant properties that can be exploited for the production of fermented beverages. Particularly, the cryotolerance and capacity to produce high amounts of volatile compounds offers new opportunities for the fermentation industry. *Kluyveromyces marxianus* is another food-grade yeast that is of interest for its aroma properties. In *S. cerevisiae*, nitrogen metabolism is well-understood but less is known about these pathways in non-traditional yeasts, nor whether there are regulatory differences between the yeasts. This thesis explores nitrogen metabolism in non-traditional yeasts and the nitrogen source effect on the metabolome and transcriptome. The main focus was on *S. uvarum* as this is already used to a certain extent in the fermented beverage sector. First, we established the nitrogen preference of *S. uvarum* and subsequently performed fermentations with a reference *S. uvarum* strain in oenological conditions varying the nitrogen source to evaluate the fermentation performance and metabolome. Next, a comparative analysis of gene expression (RNAseq) in ammonium, methionine, phenylalanine and asparagine was performed to

determine how the nitrogen source affects the expression of key genes involved in nitrogen metabolism and aroma production in this species. In overall terms, the pathways used by *S. uvarum* were equivalent to those used by *S. cerevisiae* but some detail varied. One such detail was a major difference between *S. cerevisiae* and *S. uvarum* in use of asparagine, which we found was a preferred amino acid for *S. cerevisiae* but not for *S. uvarum*. This was intriguing, in particular because asparagine is the major source of nitrogen in apple juice, where *S. uvarum* is traditionally used for cider fermentation. This led us to explore the evolution history and functional role of *ASP3*, a gene involved in asparagine assimilation. Our data on the use of amino acids by *S. uvarum* highlighted the role of the Ehrlich pathway in interconversion of nitrogen compounds and in generation of precursors of aroma volatiles. As a complement to this work in *S. uvarum*, we expanded the study of amino acid metabolism to *K. marxianus,* specifically focusing on expression of the *BAT1* gene encoding a branched chain aminotransferase responsible for both synthesis and catabolism of branched chain amino acids. The intriguing aspect of this was that while *Saccharomyces* spp. possess separate genes for the mitochondrial and cytosolic forms of the enzyme, *Kluyveromyces* spp. have a single gene that can be used to produce alternative proteoforms for the same purpose. In addition to uncovering a novel mechanism of transcriptional regulation in *K. marxianus*, the work also provided information to help explain the evolutionary basis for the retention of two copies of the gene in *Saccharomyces spp*. Overall, the research reported in this thesis demonstrates that exploring the metabolism and characteristics of non-conventional yeasts opens up possibilities to find interesting traits with potential industrial applications. This study increases understanding of the importance of the nitrogen source in fermentation performance and the flavour and aroma production of yeast and broadens the knowledge on *S. uvarum,* in particular, for applications in the fermented beverage industry.

Résumé en français

INTRODUCTION

De par ses propriétés, la levure est un micro-organisme de choix pour l'industrie, notamment pour la production de boissons fermentées où elle joue un rôle essentiel dans les secteurs du vin, de la bière et du cidre. La qualité et les caractéristiques de ces produits sont fortement modelées par les performances des levures mises en jeu, qui dépendent des conditions environnementales dans lesquelles se déroule la fermentation. Ainsi, grâce à la combinaison de connaissances sur la biologie des levures et sur le procédé de fermentation, les scientifiques peuvent améliorer les itinéraires de production et générer des boissons innovantes présentant de nouvelles propriétés.

Saccharomyces cerevisiae est l'espèce de levure la plus fréquemment rencontrée dans l'environnement et, en conséquence, la plus étudiée. Cette levure se caractérise par des propriétés d'intérêt pour la production d'aliments et de boissons fermentés, comme sa capacité de production et sa tolérance à l'éthanol, sa capacité à achever efficacement la fermentation et à produire des saveurs et arômes contribuant à la qualité sensorielle des produits, son innocuité vis-à-vis du consommateur. L'accumulation de ces caractéristiques d'intérêt technologique peut s'expliquer par le fait que l'homme a domestiqué la levure à partir d'isolats. Certaines études ont révélé comment les levures présentant des traits phénotypiques d'intérêt ont été sélectionnées au fil du temps, pour améliorer la qualité des boissons ou aliments. Ces levures sont actuellement utilisées comme starters pour l'inoculation massive des moûts, ce qui permet de fiabiliser le procédé industriel de production. Cependant, *S. cerevisiae* n'est pas la seule espèce de levure rencontrée dans l'industrie des boissons et aliments fermentés. En effet, il existe une communauté microbienne très diversifiée, à la fois dans les matières premières et sur le matériel utilisé en production - par exemple,

dans le cas de la vinification, dans les moûts de raisin et dans l'environnement de la cave- et ces microorganismes sont retrouvés dans les procédés de fermentations, où ils peuvent contribuer de façon positive ou négative. Longtemps considérées comme micro-organismes d'altération, les levures issues de ces communautés naturelles, appelées levures non traditionnelles, connaissent actuellement un regain d'intérêt dans les industries brassicole, vinicole et en panification. En effet, certaines d'entre elles montrent des propriétés spécifiques très diverses, pouvant être exploitées pour l'amélioration de la qualité des produits fermentés, notamment au niveau des profils de production de molécules issues de la fermentation : glycérol, acides organiques et composés volatils ou de leur capacité de production d'enzymes hydrolytiques participant à la révélation d'arômes variétaux. Cela est certainement lié au développement de stratégies différentes à celle de S. cerevisiae pour survivre et se développer dans les environnements peu favorables que sont les matrices alimentaires. Dans ce projet, nous nous sommes focalisés sur les levures *Saccharomyces uvarum* et *Kluyveromyces marxianus* pour lesquelles des caractéristiques intéressantes pour les procédés de fermentation industrielles ont été rapportées dans la littérature, notamment une bonne capacité de fermentation à basse température, la diversification du profil de production de composés aromatiques et une faible production d'éthanol.

L'un des sujets les plus étudiés dans les boissons fermentées est la production de métabolites volatils qui contribuent à la saveur et à l'arôme. Parmi ces métabolites, on trouve des alcools supérieurs, des esters d'acétate, des esters d'éthyle et des acides gras qui, de façon globale, sont associés à des descripteurs de type fruités et floraux, qui sont recherchés dans les boissons alcoolisées (Stribny et al., 2015). Le métabolisme secondaire de la levure est responsable de la production de ces composés ; par conséquent, le génotype de la levure est essentiel pour obtenir le profil aromatique souhaité. Néanmoins, la production de ces composés est affectée par des facteurs environnementaux, tels que la nature et la

disponibilité de la ressource azotée et la température de fermentation. La compréhension de la réponse génétique de la levure à ces facteurs environnementaux est nécessaire pour développer de nouvelles boissons fermentées ou distillées présentant des caractéristiques sensorielles particulières.

Ainsi, compte tenu de l'importance des levures dans la production de boissons fermentées, il est essentiel de comprendre le rôle et la contribution des levures d'intérêt tout au long de la fermentation. Pour cela, Querol et Fleet (2006) ont proposé deux approches : l'une est centrée sur la connaissance du procédé de fermentation. Le volet expérimental est alors dédié à analyser l'incidence de la souche de levure (ou des communautés de levure), de la composition de la matière première et d'opérations technologiques sur les performances fermentaires et la qualité des boissons fermentées. De façon plus détaillée, ce travail repose sur la mise en œuvre de méthodologies permettant de suivre l'activité des levures, permettant d'évaluer l'incidence de facteurs génétiques et environnementaux sur la cinétique de fermentation). D'autre part, grâce au développement de méthodes analytique pour la quantification des métabolites, le comportement de la levure dans diverses conditions de fermentation et les mécanismes impliqués dans la régulation de son métabolisme sont caractérisés. La deuxième approche consiste à étudier la biologie de la levure elle-même dans le contexte des boissons fermentées. Cela implique l'étude de la génomique, de l'évolution, du métabolisme et de la réponse au niveau moléculaire de la levure à son environnement. Ces connaissances sur la biologie et la physiologie des levures permettent de prédire le comportement des levures au cours de la fermentation, et ainsi de proposer des alternatives pour moduler leur métabolisme au cours de la fermentation et l'orienter vers la formation de molécules d'intérêt.

Dans ce projet de thèse, ces deux approches ont été mises en oeuvre pour étudier deux levures non traditionnelles *S. uvarum* et *K. marxianus*, qui présentent des traits phénotypiques d'intérêt technologique, notamment

au niveau de la production d'arômes. Ainsi, nous avons évalué la réponse à des modifications environnementales comme la composition de la source d'azote, de ces espèces (au niveau des performances fermentaires et de la production de métabolites), mais aussi caractérisé en détail leur métabolisme, leur profil d'expression génique et leur régulation de gènes spécifiques impliqués dans l'assimilation de l'azote et la production d'arômes.

L'étude se concentre sur trois questions principales, qui sont : (i) étudier la façon dont le génotype de la levure et les voies métaboliques interagissent avec l'environnement, en se concentrant sur l'effet de la source d'azote, (ii) explorer le comportement et le profil de croissance des levures dans les fermentations du vin et du cidre, et (iii) comprendre la fonction, l'expression et la régulation de gènes spécifiques (*ASP3* et *BAT1*) impliqués dans les voies d'assimilation de l'azote également liées à la production d'arômes. Ainsi, cette thèse vise à apporter une contribution originale à des sujets académiques importants tels que l'évolution, la régulation de l'expression des gènes et le métabolisme des levures lié à la production d'arômes. De plus, elle génère des connaissances pour les secteurs de la biotechnologie tels que l'amélioration des souches industrielles, en soulignant l'importance de levures non traditionnelles utilisée pour moduler le profil sensoriel du vin. Cette thèse fournit également de nouvelles informations sur les spécificités du réseau métabolique de *S. uvarum* en fonction des fluctuations environnementales qui se produisent pendant la fermentation du vin.

PRINCIPAUX RÉSULTATS ET DISCUSSION

Dans les chapitres 1 et 2 de cette thèse, nous avons abordé les relations entre le catabolisme des substrats azotés et le métabolome chez *S. uvarum*, afin d'expliquer les traits phénotypiques spécifiques à cette espèce.

Dans le chapitre 1, nous avons en premier lieu évalué si *S. uvarum* présentait des différences marquées avec *S. cerevisiae* dans sa capacité à utiliser

différentes sources d'azote. Pour cela, les cinétiques croissance de ces deux levures sur milieu minimal comportant différentes sources d'azote (acides aminés et ammonium) ont été comparées à 20°C. De plus, des fermentations ont été réalisées en milieu synthétique, en utilisant 9 acides aminés comme seule source d'azote et en matrice naturelle : jus de raisin blanc. Pour chaque fermentation, nous avons caractérisé les performances cinétiques de *S. uvarum* et mesuré la production de métabolites carbonés centraux et de composés volatils. Cela nous a permis de dresser un tableau complet de l'incidence des sources d'azote sur la croissance de *S. uvarum* et son comportement en fermentation. Ce travail a également permis de souligner les spécificités métaboliques de cette levure.

Nous avons en particulier observé des différences significatives de croissance entre *S. uvarum* et *S. cerevisiae* lorsque la méthionine et l'asparagine sont utilisées comme seule source d'azote. Cela s'explique probablement par mécanismes de régulation de l'utilisation de l'azote différents entre les deux espèces. D'autre part, nous avons montré que *S. uvarum*, présentait de bonnes performances fermentaires, à la fois au niveau de la cinétique fermentaire et de la capacité à consommer la totalité des sucres du milieu, lorsque l'azote est apporté sous forme d'acides aminés assimilés par la voie d'Ehrlich (valine, isoleucine, leucine, phénylalanine et méthionine). Cela s'accompagne d'une production accrue d'alcools supérieurs et d'esters d'acétate. Il est intéressant de noter que l'effet positif de ces sources d'azote sur le comportement fermentaire de *S. uvarum* s'accompagne d'un effet négatif sur sa croissance (corrélation négative).

De plus, *S. uvarum* présente une production élevée de succinate et de glycérol et une faible production d'éthanol et d'acétate en conditions de fermentation œnologique. Il s'agit d'un profil rapporté précédemment comme caractéristique des levures cryotolérantes. Cependant, nous avons mis en évidence une influence importante de la source d'azote dans la production de succinate et d'acétate. La production de ces métabolites est plus élevée lorsque la source d'azote utilisée pour la fermentation conduit à

une croissance rapide de *S. uvarum.* Cela s'explique probablement par des besoins anaboliques accrus, notamment au niveau de la synthèse *de novo* d'acides aminés protéinogéniques. Cela se traduit par des ré-orientations métaboliques pour assurer les besoins en précurseusrs, en énergie et de maintien de l'équilibre redox conduisant probablement à une augmentation de la production de succinate. De plus, l'analyse intégrée de l'ensemble de nos données apporte de nouvelles informations sur le métabolisme de l'acétate chez *S. uvarum*. Nous proposons un modèle de gestion des flux métaboliques autour de l'acétate intracellulaire chez cette espèce, qui conduit à une utilisation principale de l'acétate pour la formation d'acétyl-CoA. Cet intermédiaire est ensuite utilisé comme précurseur pour la production d'acides gras à moyenne chaine, qui sont utilisés à la fois pour la formation des membranes cellulaires et la synthèse des esters éthyliques d'ACFM en C10 et C12. Ces observations sont à mettre en regard du phénotype cryophile de cette espèce.

En ce qui concerne la question de recherche sur l'incidence de la source d'azote dans la production de composés aromatiques, les tests statistiques ont révélé que la production de certains composés volatils est fortement influencée par la présence de composés azotés, suivant des schémas qui sont spécifiques à chaque souche. De plus, nous avons identifiés des spécificités métaboliques propres à *S. uvarum* : cette espèce présente en effet une forte capacité de production d''acide 2-méthylbutanoïque et de dodécanoate d'éthyle, quelle que soit la source d'azote utilisée. Cette recherche a mis en évidence l'importance de *S. uvarum* utilisé pour moduler le profil sensoriel du vin.

Dans le chapitre 2, nous avons cherché à approfondir nos connaissances sur le métabolisme de l'azote et sa régulation chez *S. uvarum*, en étudiant comment la nature de la source d'azote pouvait interagir sur le profil d'expression génique de cette espèce. Pour cela, nous avons effectué une analyse comparative du transcriptome et du métabolome de *S. uvarum* en fonction de la source d'azote utilisée pour la croissance : ammonium,

asparagine, méthionine et phénylalanine. Les cultures ont été réalisées en milieu minimal, contenant 20 g/L de sucre et 1059 mg N/L.

Nous avons d'abord déterminé l'efficacité de ces sources d'azote à assurer la croissance de *S. uvarum* MTF3098 en comparant les valeurs de l'AUC. Nous avons constaté que l'ammonium soutient efficacement la croissance, la phénylalanine et la méthionine soutiennent une croissance intermédiaire, tandis que la croissance la plus faible a été observée avec l'asparagine. Ensuite, nous avons analysé une analyse comparative du transcriptome de *S. uvarum* en comparant les profils d'expression géniques obtenus lors de la croissance sur acide aminé (asparagine, méthionine, phénylalanine) à celui observé au cours de la culture sur ammonium. Nous avons tout d'abord mis en évidence une réponse transcriptionnelle commune aux acides aminés, qui concerne seulement un petit nombre de gènes et qui est essentiellement liée au système de transport de l'azote. D'autre part, chacun des acides aminés génère une réponse au niveau de l'expression génique qui lui est spécifique. L'asparagine, qui induit la réponse transcriptomique la plus importante en nombre de gènes, conduit notamment à une sous-expression des gènes impliqués dans la biogénèse des ribosomes, par rapport à l'ammonium. Ce comportement est à relier aux différences de croissance de *S. uvarum* sur ces deux sources d'azote. La méthionine provoque une surexpression des gènes de biosynthèse des acides aminés branchés, tandis que la phénylalanine induit de façon significative les gènes liés à la réponse au stress.

La comparaison de nos données avec les travaux de la littérature portant sur la réponse transcriptomique de *S. cerevisiae* à la nature de la source d'azote montre que ces deux espèces régulent de façon similaire la consommation et le catabolisme des sources d'azote. Cependant, il est possible que l'activité intrinsèque des protéines soit spécifique à *S. uvarum*, générant un phénotype différent. De plus, les données sur le métabolome de *S. uvarum* ont établi l'existence d'une relation entre la production de certains composés volatils et la disponibilité de l'acide aminé précurseur de leur

biosynthèse. L'augmentation de la formation de ces composés volatils est également liée à une surexpression des gènes impliqués dans leur formation, comme les gènes de la voie d'Ehrlich, *ADH*s et *ALDs* surexprimés lors des cultures sur méthionine et phénylalanine. Nos résultats suggèrent que la régulation de gènes spécifiques a des conséquences importantes sur le métabolome de *S. uvarum*, notamment sur la production de composés volatils.

Ce chapitre a montré que des modifications du profil d'expression géniques étaient impliquées dans la modulation de la production des composés aromatiques par la source d'azote. Pour aller plus loin dans la compréhension mécanismes moléculaires sous-jacents aux spécificités phénotypiques de *S. uvarum*, une analyse détaillée des différences entre *S. cerevisiae* et *S. uvarum* au niveau des séquences génomiques et du niveau d'expression de gènes cibles doit être menée.

Le chapitre 3 de ce projet a été dédié à comprendre les différences de capacité de croissance entre *S. cerevisiae* et *S. uvarum* lorsque l'asparagine est utilisée comme seule source d'azote, observées dans les chapitres 1 et 2. Nous avons émis l'hypothèse que la différence d'efficacité de croissance pourrait être liée à la présence du gène *ASP3*, codant pour l'asparaginase II qui est impliquée dans la dégradation de l'asparagine. Cette enzyme, dont l'activité peut être extracellulaire, catalyse l'hydrolyse de l'asparagine en aspartate et ammonium. Nous avons constaté que la plupart des souches du genre *Saccharomyces* ne possédaient pas ce gène, à l'exception de certaines souches de *S. cerevisiae*. En particulier, *ASP3* est absent des souches de *S. uvarum* que nous avons caractérisées.

Afin de déterminer si la présence de ce gène est liée à l'adaptation des levures à leur niche écologique, nous avons étudié le rôle et l'histoire évolutive de *ASP3*. Nous avons construit une base de données regroupant les séquences génomiques de 1680 souches de *S. cerevisiae* dont l'origine écologique était décrite. La présence d'au moins une copie d'*ASP3* a été détectée dans le génome de 117 souches (~7%). De façon surprenante, nous

avons trouvé une distribution dispersée d'*ASP3* sur l'arbre phylogénétique alors que sa présence sur un clade distinct, reflétant une acquisition de ce gène par un seul événement de transfert horizontal de gènes (THG), était attendue. De plus, l'analyse de la synténie au sein des souches de *S. cerevisiae* a révélé la présence de locus conservés. L'analyse phylogénétique et de synténie de l'*ASP3* chez d'autres espèces de levures ne montre aucune synténie pour le gène *ASP3*. Par conséquent, nous proposons qu'*ASP3* est un gène ancestral chez *S. cerevisiae*, et qu'il a été perdu plusieurs fois au cours de l'évolution, mais qu'il a été conservé dans certaines souches. Nos données infirment l'acquisition de ce gène par transfert horizontal, précédemment proposée (League et al., 2012).

Sur la base de cette analyse de l'origine et de l'évolution du gène *ASP3* chez *S. cerevisiae*, nous avons orienté nos recherches vers la compréhension de l'avantage de ce gène dans les environnements fermentaires. Notre approche a consisté à évaluer l'expression hétérologue d'*ASP3* chez *S. uvarum*. Cette espèce de levure, dépourvue du gène *ASP3* se retrouve dans les mêmes environnements que *S. cerevisiae* : ainsi, la conservation du gène *ASP3* pourrait conférer un avantage sélectif aux souches de *S. cerevisiae*. Nous avons montré que la présence du gène *ASP3* chez *S. uvarum* permettait à cette levure d'améliorer ses performances de croissance en présence d'asparagine en tant que seule source d'azote. Cependant, ce résultat n'explique pas la différence de croissance entre *S. cerevisiae* et *S. uvarum* observée dans les chapitres 1 et 2, car la souche EC1118 de *S. cerevisiae* utilisée pour les fermentations du vin ne possède pas le gène *ASP3* dans son génome, mais montre une croissance efficace sur asparagine.

De plus, afin d'établir si la présence du gène *ASP3* confère un avantage sélectif au cours de fermentation en milieu naturel riche en asparagine, comme le jus de pomme, nous avons comparé les performances fermentaires d'une souche de *S. uvarum* surexprimant *ASP3* à celles d'une souche sauvage en fermentation cidricole. Aucun avantage sélectif de *ASP3* n'a été mis en évidence dans ces conditions. Cependant, cette étude a

permis de mieux comprendre les facteurs affectant l'activité métabolique portée par *ASP3*. En effet, la croissance de *S. uvarum* est fortement améliorée par la présence du gène *ASP3* lorsque l'asparagine est la seule source d'azote disponible, ce qui peut être expliqué par l'hydrolyse extracellulaire de l'asparagine en aspartate et ammonium via Asp3p, ces deux dernières sources d'azote étant plus facilement transportées que l'aspargine. A l'inverse, aucun effet lié à la présence de ce gène n'est observé lors de culture en présence d'une source d'azote plus complexe (mélange d'acides aminés). Ces observations nous ont conduit à proposer que la modification du profil d'assimilation des composés azotés, via la régulation des transporteurs, liée à la présence d'une source d'azote mixte, masquant l'avantage de l'activité de *ASP3.*

Nous nous sommes intéressés dans le quatrième chapitre de cette thèse à la régulation transcriptionnelle du gène *BAT1*, qui est impliqué dans la conversion des acides aminés à chaîne ramifiée (BCAA). Nous avons exploré les mécanismes impliqués dans la formation des isoformes cytoplasmique et mitochondriale de l'aminotransférase des acides aminés à chaîne ramifiée, à partir d'un gène unique *BAT1* et en utilisant des sites alternatifs de d'initiation de transcription (aSIT) chez la levure ancestrale *Kluyveromyces marxianus*. Une approche holistique a été utilisée, combinant l'étude de l'expression et de la localisation des gènes avec la caractérisation de l'effet de mutations génétiques sur la croissance, pour établir le mécanisme d'utilisation des sites alternatifs de d'initiation de transcription en fonction des conditions de culture. Tout d'abord, grâce à une analyse *in silico* des sites d'initiation de transcription (SIT), des données d'expression génique et des données sur le profilage des ribosomes, nous avons formulé l'hypothèse selon laquelle *K. marxianus* utilise l'aSIT pour produire des protéoformes cytosoliques ou mitochondriales de la BCAT. Nous avons proposé que lorsque la biosynthèse des BCAA est nécessaire, la transcription s'initie au premier SIT donnant lieu à une protéoforme plus longue contenant un signal de ciblage mitochondrial), alors que lorsque les BCAA sont en excès, le

second SIT est utilisé, produisant une protéoforme cytosolique plus courte. Ensuite, grâce à des méthodes de localisation des protéines et d'expression des gènes, nous avons constaté qu'en présence de BCAA, il n'y a pratiquement pas de transcription à partir du premier SIT. Seul le second SIT est utilisé, et en conséquence seule la protéoforme cytosolique est produite. En revanche, dans des conditions de biosynthèse, lorsque la seule source d'azote est l'ammonium, les deux sites d'initiation de la transcription sont utilisés de manière égale, ce qui conduit à la synthèse des protéoformes mitochondriales et cytosoliques.

Ayant établi que la synthèse des deux protéoformes est contrôlée au niveau transcriptionnel, nous avons identifié les facteurs de transcription (TFs) qui pourraient être responsables du contrôle transcriptionnel. Pour cela, nous avons généré des mutants de délétion de huit TFs chez *K. marxianus* (*GAT1, GCN4, GLN3, HAP2, MOT3, NRG1, PUT3, LEU3*). La caractérisation de ces mutants montre que, comme chez *S. cerevisiae*, Gcn4p et Leu3p favorisent l'expression de l'ARNm codant pour la protéoforme mitochondriale dans des conditions où la synthèse des BCAA était requise. Cependant, contrairement à *S. cerevisiae*, *K. marxianus* n'a pas de régulation fine de l'ARNm codant pour la protéoforme cytosolique. De façon globale, nos données vont dans le sens d'un modèle évolutif pour lequel les levures ayant subi l'événement de duplication du génome entier (WGD), ont maintenu la plupart de la régulation ancestrale dans une copie d'un gène dupliqué et ont adapté les facteurs existants agissant en cis et en trans pour développer un nouveau cadre de régulation pour la seconde copie.

CONCLUSIONS

Cette thèse a contribué aux connaissances existantes du secteur de l'innovation des boissons fermentées et de la recherche sur le métabolisme des levures. Notre étude sur les levures non traditionnelles a démontré que la nature de la source d'azote influence la croissance, les performances

fermentaires et la production de saveurs et d'arômes, en lien avec le génotype de la levure. Nous avons notamment apporté un éclairage nouveau sur le métabolisme des arômes au niveau génomique, transcriptomique et métabolomique et mis en évidence des spécificités à la fois phénotypiques mais aussi génomiques et transcriptionnelles (études *ASP3* et *BAT1*) des espèces non traditionnelles par rapport à la levure modèle *S. cerevisiae*. Dans l'ensemble, ces résultats fournissent des connaissances essentielles pour exploiter le potentiel des levures non conventionnelles dans le secteur de l'industrie alimentaire et des boissons, notamment pour développer de nouvelles boissons fermentées. Ainsi, l'utilisation de souche sélectionnées, associée à une gestion appropriée des nutriments, peut avoir un impact important sur la qualité des boissons fermentées, notamment sur le profil organoleptique. D'autres paramètres, tels que la croissance des levures et la cinétique fermentaire, peuvent être optimisés par le biais de la sélection de la souche et la gestion de la nutrition azotée. En conséquence, ces informations pourront être appliquées par les industriels dans la sélection des souches (présence de gènes favorisant un phénotype recherché) à utiliser et la gestion du procédé de fermentation pour la production de boissons fermentées.

Introduction

1. General introduction

The properties of yeast cells make them one of the most advantageous microorganisms in industry, especially in the production of fermented beverages (Maicas, 2020). In particular, yeast has been used from the ancient times for wine and beer making. The quality and characteristics of the final product depend on the yeast performance and the environmental conditions during fermentation. Combining knowledge of yeast biology and the different fermentation elements allow scientists to generate innovative beverages with new properties and improved processes. Although *Saccharomyces cerevisiae* is the most important and most widely used yeast species, other non-traditional yeasts are attracting industrial interest because of the new traits of technological interest that they offer.

Fermentation is a process carried out by yeast, where the sugar is converted to ethanol and other by-products. This process consists of a lag phase for yeast adaptation to the environmental conditions, a growth phase, where the cells reproduce and consume sugar and a stationary phase, where there is a balance of cells duplication and dead. During the stationary phase the sugar is depleted and the production of secondary metabolites takes place. The monitoring of the process is done by measuring substrate consumption and product generation, and the yeast growth is usually monitored by measuring the CO_2 produced and the population size. By this practice, raw materials get converted in desired products categorized as fermented food and beverages. Moreover, one of the most investigated topics in fermented beverage is the production of volatile metabolites that contribute to flavour and aroma. Among these metabolites, there are higher alcohols and acetate esters that contribute to the fruity and floral aromas desired in fermented beverages (Stribny et al., 2015). The production of these compounds is affected, beyond the yeast strain that carries out the fermentation, by

external factors, such as nitrogen source and temperature of fermentation. The understanding of the yeast genetic response to these environmental factors linked to flavour and aroma is needed to develop novel fermented or distilled beverages with special characteristics.

Knowing the importance of yeast in the production of fermented beverage, we need to focus on the approach to understand the yeast role in fermentation. For this, Querol and Fleet, (2006) proposed two approaches; one is centred on the technology and fermentation process, thereby, the experiments consist on exploring the impact of different yeast strains, raw materials, and environmental factors on fermentation performance and quality of fermented beverages. The second approach is to investigate the yeast biology itself in a fermented beverage context. This involves the study of the yeast genomics, evolution, metabolism, and molecular response to the environment. Knowledge of the fundamental yeast biology helps to predict the yeast behaviour in the fermentation process and gives alternatives to modulate the fermentation and obtain the desired product. Most of these studies have focused on *S. cerevisiae*, while other yeast species remain less-well explored and characterised.

In this thesis, the two aforementioned approaches were conducted to study non-traditional yeasts with attractive flavour and aroma traits. *Saccharomyces uvarum* was used in this work because of its commercial advantages and current attractive applications in the fermented beverage industry. On the other side, we focused on *Kluyveromyces marxianus* since this is a non-conventional yeast with potential properties to be exploited in biotechnology sector. These yeasts were compared to the model yeast *S. cerevisiae* in order to understand their mechanisms. The study focuses on three main topics, which are: (i) understanding the function, expression and evolution of specific genes related to aroma production (*ASP3* and *BAT1*), (ii) exploring the non-traditional yeast behaviour and growth profile in wine and cider fermentations, and (iii) investigating how the yeast genotype and metabolic pathways interact with the environment. Thus, this thesis intends

to make an original contribution to important academic subjects such as, evolution, gene expression regulation, and yeast metabolism, as well as generating knowledge for biotechnology sectors such as industrial strain improvement.

2. Yeast biotechnology

Yeasts are unicellular fungi classified in the *Ascomycetes* phylum, recognized for their ability to ferment sugars, and produce ethanol and CO_2. These microorganisms are found in different environments such as soil, leaves, fruits, water, human skin, and intestinal tract. The most well-known yeast species is *S. cerevisiae* (Goffeau et al., 1996; Liti, 2015), which during the last decades has been the model system for genetic research due to its conserved cellular mechanics with larger eukaryotes, including mammals (Schneiter, 2004). Additionally, *S. cerevisiae* and related species have been used in industry to ferment sugars present in rice, wheat, barley, corn, and fruits, and beer and wine making are the industries with major impact. Besides, there are other yeast species that positively impact industrial fermentations, referred as non-cerevisiae yeasts (Holt et al., 2018). Some non-cerevisiae strains have been isolated and identified from organic substrates and are now commercialised after a careful observation of competitive value and high fermentation performance (Marullo and Dubourdieu, 2010; Rollero et al., 2018). The use of those strains requires optimization of the fermentation conditions such as media composition and environmental parameters.

Yeast strains can also be manipulated to improve the phenotype and increase production levels of desired metabolites. Normally, in the fermented food and beverage industries this is done either with non-genetically modified (non-GM) methods such as mutagenesis, hybridization or adaptive evolution that select for special yeast traits (Giannakou et al., 2020; Gibson et al., 2017). By contrast, there are GM methods that use genetic engineering to improve functions or introduce new traits in the

organism, by altering the genome (Committee on Identifying and Assessing Unintended et al., 2004). The latter is used in cosmetics, pharmaceutic and white biotechnology sectors, where the entire genetically modified organism (GMO) is not in contact with the consumer (Schindler, 2020; Schuller and Casal, 2005). In this regard, the use of yeast cell factories stands out because yeast is well characterised, is easy to cultivate, grows fast and, most importantly, the genome sequence is well-known and accessible leading to easy genetic manipulation. One of the most important applications is recombinant protein production, which uses yeast as the production host of valuable proteins (Mattanovich et al., 2012).

2.1. Applications in fermented beverages

2.1.1. Wine

Wine production is linked to the history of agriculture, dating back thousands of years. Winemaking technology is widespread worldwide and has been improved since the discovery of yeast in 1680 (Chambers and Pretorius, 2010). Wine has an important economic and cultural value; therefore, wine practices keep being optimized and enormous scientific work has been done to understand the role of grapes, yeast, and techniques in wine production. With the improvement of wine practices, the development of wine strains occurred, also defined as yeast domestication. Since 1890, pure yeast starter cultures have been inoculated in grape juice, increasing the control and consistency of the fermentation process (Marsit and Dequin, 2015). However, native yeasts are still used to produce wine with traditional oenological practices. The focus on these practices and the use of genomic approaches to characterise the natural microbiota, allowed the discovery of several non-conventional yeast strains with interesting traits for wine production. In a general point of view, it has been reported that the major factors affecting the wine quality are the grape variety, viticultural practices, and fermentation methods, nonetheless, yeast is considered to have the main role in winemaking.

The grape must is a complex growth medium that contains "Yeast Assimilable Nitrogen" (YAN) in concentrations between 60 and 500 mg/L (Bely et al., 1990; Ugliano et al., 2007), therefore, nitrogen can be considered a limiting nutrient for wine fermentation. Additionally, wine must has low pH and high concentrations of sugars that serve as substrate for yeast and many other microorganisms. However, the number of microbial species that are able to grow in grape must is low, resulting in yeast being the principal biochemical convertor. The low pH is one of the factors that limits the growth of non-yeast microorganisms. In addition, factors associated to the fermentation conditions that increase the selectivity of microbial community in the medium are low pH, high sugar content, addition of sulphur dioxide and absence of oxygen. Other growth limiting factors observed during the second stage of fermentation caused by yeast, are the high amount of ethanol and low availability of nutrients including nitrogen and some vitamins (Ciani et al., 2010; Renault et al., 2013). *S. cerevisiae* is well adapted to these constraints, exhibiting tolerance to ethanol and other stressors. In fact, *S. cerevisiae* is the major ethanol producer in wine, causing growth decline of non-cerevisiae species when the ethanol produced reaches more than 7% in the fermentation. Despite this, non-cerevisiae species do make positive contributions in traditional wine fermentation, with roles described for other *Saccharomyces spp*., *Hanseniaspora sp. (Kloeckera), Torulaspora sp., Metschnikowia spp.,* and *Kluyveromyces spp.* (Comitini et al., 2011; Viana et al., 2008).

2.1.2. Cider

Cider is an alcoholic beverage obtained from the fermentation of apples. This drink is well-known around Europe, although the cider market is also important in Australia and North America. Although, relatively little is currently known about factors affecting apple juice fermentations, some research has been done on apple juice composition and the yeast species used in the fermentation process (Magalhães et al., 2017). It is common to find equivalent concentrations of fructose and glucose, however, some

slight variations in the ratio fructose/glucose may be found depending on the apple type. The nitrogen content in apple musts varies between 50 and 400 mg/L of free amino nitrogen (FAN), with asparagine as the most abundant amino acid in apple juice. The quantity of asparagine in apple juice is 100 times higher than in grape juice (Dizy et al., 1992; Ma et al., 2018). It has been found that the variety and ripening stage of apples influence the flavour and aroma profile of cider (Rosend et al., 2019). Nevertheless, the aroma profile depends on the yeast strain used for fermentation. Finally, relating to yeast diversity in cider, the most common wild yeasts found in the apple must are *Saccharomyces spp*., mainly *S. uvarum, Hanseniaspora spp.* or other species with low fermentation activity, such as *Candida spp., Pichia spp., Metschnikowia spp.* and *Torulaspora spp*. Most of these species display a cryophilic profile, as the fermentation of traditional cider is made at temperatures between 12°C and 16°C. The orchards, yeast strains and cider making technologies are all associated with cold environments, which elongates the fermentation process.

2.1.3. Beer

In brewing, the most common yeasts species are *S. cerevisiae* and *Saccharomyces pastorianus,* classified as Ale and Lager species, respectively (Gibson et al., 2017; Rose and Harrison, 1993). Ale yeasts are top-fermenting yeast that do not ferment melibiose and can grow at approximately 37°C, in contrast, Lager yeasts are bottom-fermenting yeast that grow at low temperatures and ferment melibiose due to the presence of the melibiase enzyme (Querol and Fleet, 2006). In beer making, the substrate is malted barley, which contains sugars such as maltose (the most dominant), maltotriose, glucose, sucrose and fructose, and the addition of adjuncts influences the sugar composition of the wort. The most important compounds in beer (CO_2, ethanol, and secondary metabolites) are produced by yeast providing flavour and mouthfeel properties, which are of high interest for the brewing industry. He et al. (2014) described the influence of the wort components in the final flavour and aroma of beer and highlighted

that the nitrogen source, not only level but also type, is one of the most important factors affecting flavour and aroma of beer. For instance, the addition of amino acids like leucine, isoleucine, alanine and proline to the wort increased the production of higher alcohols (Engan, 1970). Wort contains between 140 – 280 mg/L of free amino nitrogen (FAN) depending on the wort gravity and the FAN requirements by yeast are strain specific to a certain extent (Lei et al., 2012; Saerens et al., 2008a).

3. Yeast diversity (Non-traditional yeast)

In the last decades, omics studies and modern molecular techniques have provided information about the yeast population and their evolution history in fermentation environments. Several researchers reported that species of the *Saccharomyces* genus are the most used for alcoholic beverage fermentations (Fleet, 2008; González Flores et al., 2017; Marsit and Dequin, 2015). As mentioned above, *S. cerevisiae* is the most studied and commercially used species for fermented beverages. In wine making for instance, *S. cerevisiae* dominates the fermentations due to its abilities to ferment sugars and grow in presence of high concentrations of ethanol, therefore it has been used as the starter culture by winemakers (Fleet, 2008). However, *S. cerevisiae* is not the sole microorganism involved in the fermentation. In fact, there is a large microbial community found in the grape must and the winery environment (Figure 1). Although some species are considered as contaminants of wine fermentation, some of these microorganisms could influence positively the dominant fermenter yeasts, for example, the dead cells can be source of nitrogen and can also provide enzymatic hydrolysis of uncommon components. The development of culture-independent techniques allowed the discovery and characterization of these yeast species (Cocolin et al., 2000; Masneuf-Pomarede et al., 2016a). This group of yeast, called non-traditional yeasts, are different from *S. cerevisiae* and include *Hanseniaspora sp., Pichia sp., Candida sp., Metschnikowia sp., Torulaspora sp., Starmerella sp., Kluyveromyces sp.* and other *Saccharomyces* species (Fleet, 2008). Although extensive research has

been done in *S. cerevisiae* to unravel the aroma production metabolism and the applications in alcoholic beverage, much less is known about aroma metabolism in non-traditional yeasts. In this work, we focused in two non-traditional yeasts with attractive traits for the fermented beverage industry, one belonging, and another not belonging, to the *Saccharomyces* genus.

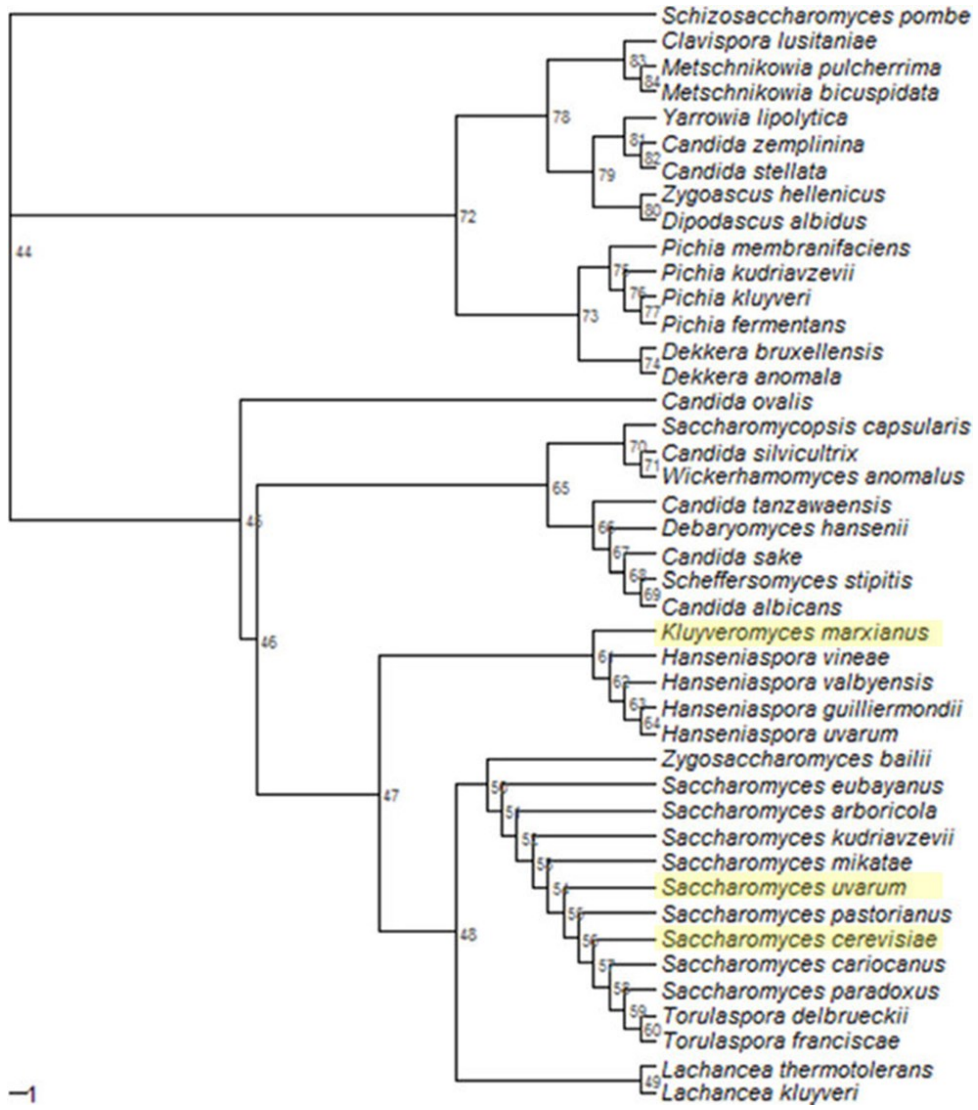

Figure 1. Phylogeny of wine yeast strains. Phylogenetic tree 41 species of Saccharomycetales constructed with the 18S ribosomal DNA sequence. The species of interest of this study are highlighted in yellow. *Schizosaccharomyces pombe* was used as outgroup species. Figure adapted from Masneuf-Pomarede et al., (2016).

3.1. *Saccharomyces uvarum*

S. uvarum is a non-traditional yeast with interesting fermentation properties. This yeast shows novel characteristics for the production of wine

and cider, even though it is genetically related to *S. cerevisiae* (Masneuf-Pomarède et al., 2010). For a period, this species was considered a synonym of *Saccharomyces bayanus* but Nguyen and Gaillardin (2005) established that *S. uvarum* is genetically isolated from *S. bayanus,* allowing the reinstatement of *S. uvarum* as a distinct species (Figure 1).

This species has been isolated from wine and cider fermentations carried on at low temperatures and is the main yeast species used for cider making. *S. uvarum* is characterised as a cryotolerant yeast, as it exhibits a good fermentation performance at low temperatures (12°C to 20°C). This trait is of special interest for white wine and cider production, as traditionally, these beverages are fermented at low temperatures. Another advantage of the cryotolerant yeast, is the fermentation profile. It has been described that cryotolerant yeasts have high glycerol and succinate production (González Flores et al., 2017; Minebois et al., 2020), which influences the beverage's body and mouthfeel. Additionally, *S. uvarum* produces low amounts of acetic acid, which is an undesired product in wine (Kelly et al., 2020; Minebois et al., 2020). More importantly, *S. uvarum* shows high production of flavour and aroma compounds. When the fermentation performance of *S. uvarum* in wine was compared to fermentations done with *S. cerevisiae* and other *Saccharomyces* strains, the properties attributed to *S. uvarum* were lower ethanol production and stronger aromatic intensity (Lopez-Malo et al., 2013; Pérez-Torrado et al., 2015). Likewise, the differences of particular orthologous genes involved in aroma production and expression level among these *Saccharomyces* species, had an impact in the aroma profile (Stribny et al., 2016). It has been reported that *S. uvarum* strains in general have higher production of acetate esters than fusel alcohols (Stribny et al., 2016, 2015). However, the high production of volatile molecules not always has a positive effect in fermented beverages. Varela et al., (2017) described the sensory profile of wines co-inoculated with *S. uvarum* and showed that the sensory attributes with the highest scores were barnyard and meat aroma. Regarding the genomic features compared to *S. cerevisiae*,

microsatellite analysis revealed that the *S. uvarum* life cycle is more closely related to *S. paradoxus* or *S. cerevisiae* of natural environments than *S. cerevisiae* isolated from wine (Masneuf-Pomarede et al., 2016b).

3.2. *Kluyveromyces marxianus*

K. marxianus has different applications in the biotechnology sector (Fonseca et al. 2008) and many strains are generally-regarded-as-safe (GRAS). It also has European Food Safety Authority (EFSA) QPS (Qualified Presumption of Safety) status and so can be used in the food and beverage industry with few restrictions. Only 2% of yeasts are able to ferment lactose, and *K. marxianus* is one of them, thus it is used in the production of fermented dairy products. One of the most important features of *K. marxianus* is a high growth rate on a broad variety of substrates in short time. Another important feature of this species is that it is very sensitive to the environmental conditions, showing different growth kinetics between strains even if the same strain is examined in different laboratories (Fonseca et al., 2007; Lane et al., 2011). The ability to grow at high temperatures with a maximum growth temperature of 52°C (Banat, Nigam and Marchant, 1992) is important for some applications, although the highest ethanol production was reported to be at lower temperatures (Anderson et al., 1986). In contrast to *S. cerevisiae*, ethanol production in *K. marxianus* was reported to decrease on sugar excess and the ethanol tolerance is low, attributed to the different activity of the plasma membrane (Rosa and Sá-Correia, 1992). The ethanol production of *K. marxianus* is linked to oxygen limitation (Van Dijken et al., 1993), although it cannot grow in complete absence of oxygen (Bellaver *et al.*, 2004). *K. marxianus* produces big amounts of fusel alcohols, acetate esters and shows high pectinase activity (Erasmus and Divol, 2022; Gethins et al., 2015), hence some strains are being explored to improve flavour and aroma in fermented food and beverages. Additionally, this trait makes *K. marxianus* an attractive cell factory for fragrances (Morrissey *et al.* 2015). Its improvement and commercial production can be achieved by understanding the metabolic pathways and gene regulation in this yeast. Concerning the genomic

characteristics of this species, evolution studies have found that the whole genome duplication (WGD) of *S. cerevisiae* happened after it diverged from *Kluyveromyces sp.* (Figure 1), so this genus is considered ancestral or pre-WGD yeast (Seoighe and Wolfe, 1999; Wolfe and Shields, 1997). Therefore, *K. marxianus* and *K. lactis* are commonly used for genetic studies to understand ancestral gene arrangements, regulation and function of *Saccharomyces spp.*

4. Yeast metabolism during fermentation

4.1. Central carbon metabolism

Microorganisms like yeast normally obtain energy from a carbon source transformation process called oxidative phosphorylation or respiration, which is done in the presence of oxygen. During respiration, one molecule of glucose is theoretically converted to 38 molecules of ATP (adenosine triphosphate) and oxygen is the final electron acceptor in the mitochondria, though the actual yield in *Saccharomyces* is lower due to the loss of complex I during evolution. In the absence of oxygen or when the sugar content exceeds 0.1% w/v, yeast implements a fermentative metabolism. The process of fermentation in yeast is done anaerobically to obtain 2 molecules of ATP per molecule of glucose consumed through glycolysis. This oxidation of glucose to pyruvate uses NAD+ as the final electron acceptor generating NADH. Then, NADH needs to be re-oxidized in the absence of oxygen (fermentation), which is done through the reduction of pyruvate to ethanol, where the main biochemical reaction is the cleavage of pyruvate by the pyruvate decarboxylase to produce CO_2 and acetaldehyde, which later is reduced to ethanol by an alcohol dehydrogenase. The conversion produces NAD+ and organic products like ethanol and CO_2, which are yeast by-products that can become toxic for the cell. Furthermore, the ethanol yield during fermentation has been reported in *S. cerevisiae,* being able to ferment its own weight of glucose per hour producing up to 18% volume of ethanol (Schneiter, 2004). The theoretical yield is 0.51 grams of ethanol per gram of glucose. This is a valuable trait of yeast because cheap substrates

can be converted into more valuable products through the fermentation process (Maicas, 2020).

In parallel to the glycolysis and ethanol production, formation of biomass precursors such as amino acids and lipids take place. Some of the pyruvate obtained from glycolysis is directed to the Tricarboxylic acid (TCA) cycle for the production of α-ketoglutarate, a key intermediate of carbon and nitrogen metabolism, that leads to the biosynthesis of amino acids subsequently used for biomass formation. In addition, another carbon metabolic route for biomass formation is the production of acetyl-CoA (via acetate) essential for lipid synthesis. These parallel routes generate an excess of NADH that needs to be re-oxidized for cell redox balance. The main identified pathway for NADH re-oxidation is glycerol generation. The main reactions taking place during fermentation are described in Figure 2.

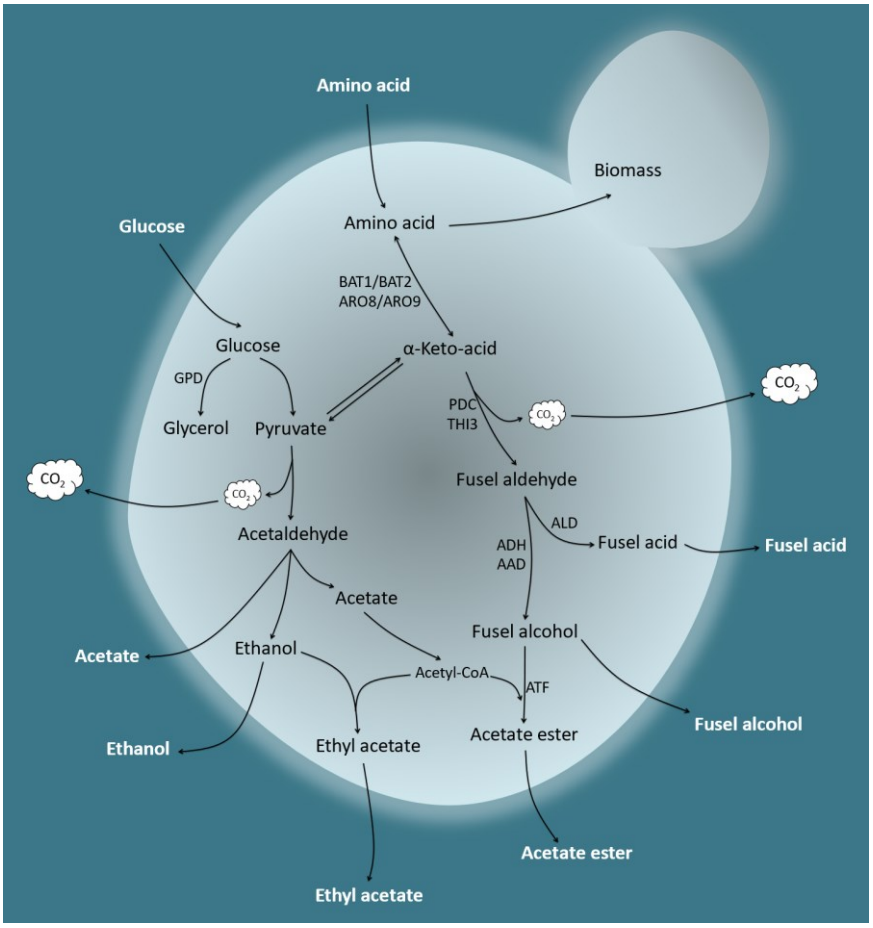

Figure 2. Main metabolic routes for aroma production in yeast during fermentation. Yeast uses specific and general permeases to import sugars and amino acids. Once inside the cell, these nutrients are catabolised, generating intermediate metabolites and CO_2. Pyruvate, α-

ketoglutarate and α-ketoacids are connecting points of the carbon and nitrogen metabolisms. The final products of the catabolic pathways are released outside the cell, most of them are volatile compounds that are associated to various aromas.

4.2. Nitrogen metabolism

The nitrogen source utilised by yeast includes ammonium salts, amino acids, and peptides. During alcoholic fermentations, amino acids are the main source of nitrogen that, once inside the cell, can have different fates depending on the yeast's requirements. The three main fates of amino acids are (i) the direct incorporation for protein formation where the anabolic metabolism uses amino acids from the media to generate biomass and proteins involved in different processes; (ii) the storage in vacuoles to be utilised in later cell phases and (iii) the catabolism in the cytosol to obtain the amino group and the carbon skeleton, needed for several purposes, including the *de novo* synthesis of amino acids. It is worth mentioning that most of the data presented in this section comes from studies done with *S. cerevisiae* and this may not fully reflect the situation in other yeasts.

4.2.1. Nitrogen transport

The transit of all kinds of molecules inside and outside the cell is required for many processes such as balancing the concentration of metabolites, nutrient uptake, secretion of toxic products, cell signalling and genetic exchange (Singer and Nicolson, 1972). The movement is facilitated by transport systems of narrow and broad range substrate specificity (Figure 2). Nitrogen molecules, particularly amino acids and ammonium are crucial for cell growth and survival, therefore the transport of amino acids is specialized and carefully regulated. Amino acid transport happens through the plasma membrane and, within the cell, through the mitochondrial and vacuolar membranes. This transport system is composed by channels, also called pores, and carriers, also called transporters or permeases. These proteins enable the change of the location of the molecule without modifying the chemical structure.

Although yeast is able to synthetise *de novo* amino acids, the import of some amino acids from extracellular environment is energetically less expensive (Wagner, 2005), thus, the amino acid transport system allows yeast to take advantage of available sources. The amino acid transporters in the plasma membrane with broad range are Gap1 and Agp1 that are involved in the transport of all amino acids in yeast, with few exceptions (Andréasson et al., 2004; Jauniaux and Grenson, 1990). Gap1 shows a higher affinity to all amino acids than Agp1. Bianchi et al., (2019) categorized the plasma membrane transporters with limited amino acid specificity in terms of the type of the substrate (amino acid), although the substrates sometimes overlap: the transporters of neutral amino acids are Agp2/3, Bap2/3, Gnp1, Mup1/3, Tat1/2/3 and Put4. Other transporters with more stringent specificity for basic amino acids are Hip1, Can1, Alp1 and Lyp1. Finally, Dip5 has high affinity for acidic amino acids, although it can also transport glycine and alanine. Regarding the transport of ammonium through the plasma membrane, the permeases Mep1/2/3 display high affinity for NH_4, of which Mep2 shows the highest affinity (Marini et al., 1997; Soupene et al., 2001).

Within the cell, the amino acids transporters (import and export) localised in the vacuole membrane, rely on electrochemical proton gradient at the expense of ATP (Russnak et al., 2001). In general, only basic amino acids such as arginine and lysine are transported in vacuoles (Crépin et al., 2014). Some of the vacuole transporters are Atg22, Vba1/2/3, Avt1/3/4/6/7, Ers1 and Ypq1/2/3 (Bianchi et al., 2019). The mitochondrial transporters translocate amino acids through the inner membrane to control the synthesis or catabolism of the substrate, depending on the location of specific enzymes (Palmieri and Pierri, 2010). The mitochondrial transporters are Agc1, Hem25, Ort1 and Sam5.

Overall, the nitrogen transport system influences the aroma volatiles production by controlling the availability of their precursors. Nevertheless, complementary transport mechanisms associated to the export of several molecules affect the aroma production by yeast. Volatile by-products in high

concentrations can be toxic for the cell, so they are easily released by simple passive diffusion without energy expense or by membrane transporters such as Pdr12 (Hazelwood et al., 2008). However, although they derive from nitrogen metabolism, the released aroma volatile molecules themselves are not nitrogen compounds.

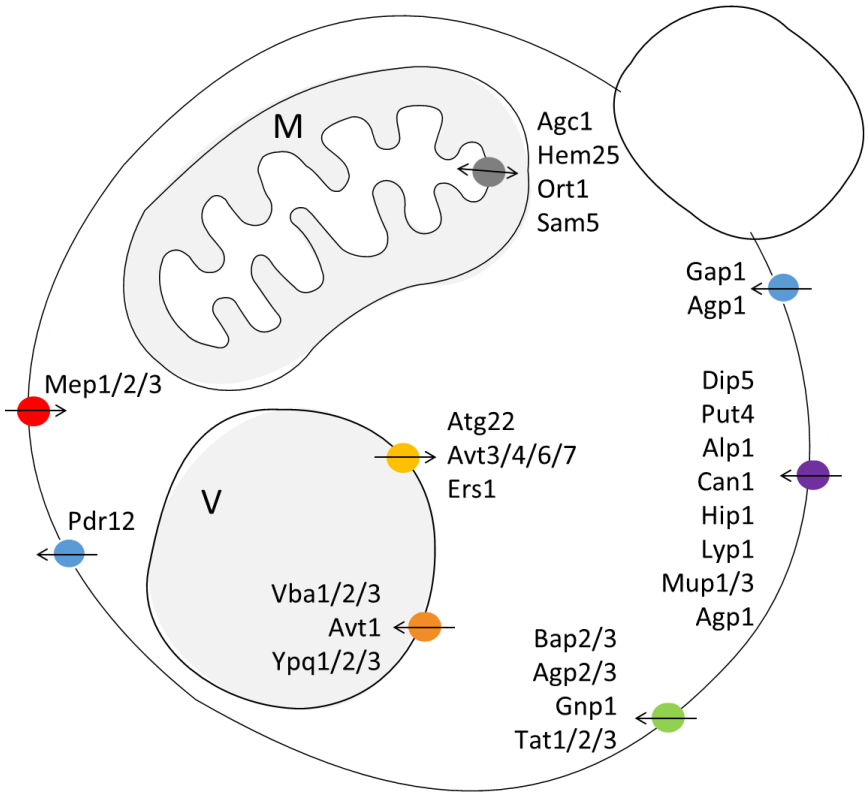

Figure 2. Nitrogen transport system. Permeases and transporters of nitrogen compounds localised is the plasma membrane, the vacuole (V) and the mitochondrion (M). Blue for broad range amino acid permeases, purple for amino acid transporters of limited specificity, green for amino acid transporters with stringent specificity, red for ammonium transporters, yellow for vacuole exporters, orange for vacuole importers, grey for inner membrane mitochondrial antiporters. Figure adapted from Bianchi et al., (2019).

4.2.2. Catabolism and anabolism of amino acids

The catabolism of amino acids is done through transamination or hydrolysis, in which the amino group is transferred to α-ketoglutarate to form glutamate, or to obtain ammonium (NH_4), releasing α-keto acid intermediates. Glutamate and ammonium together with α-ketoglutarate that derives from the central carbon metabolism (CCM) and glutamine, compose the central core of nitrogen metabolism. This pathway catalyzes

the interconversion of these compounds through an enzymatic system comprising a glutamate dehydrogenase, a glutamine synthetase and a glutamate synthase (Figure 3). The nitrogen central core is the intersection of the catabolic and anabolic nitrogen metabolisms, and also the main connection to the central carbon metabolism, through α-ketoglutarate. Furthermore, the fate of the α-ketoacids obtained from the amino acids catabolism depends on their nature. For instance, pyruvate, oxaloacetate and α-ketoglutarate are fed to the central carbon metabolism through the TCA cycle. Other α-ketoacids coming from either branched chain amino acids (BCAA) or central carbon metabolism such as α-ketoisovalerate, α-ketomethylvalerate and α-ketoisocaproate, are precursors of volatile compounds formation through the Ehrlich pathway. On the other hand, the *de novo* synthesis of amino acids takes place in the mitochondrion. The precursors for amino acids formation are α-ketoacids that can come from the catabolism of other amino acids or from the CCM, specifically, from glycolysis (pyruvate), from the pentose phosphate pathway for aromatic amino acids, and from the TCA cycle (oxaloacetate, α-ketoglutarate). Then, *de novo* amino acids are generally obtained by transamination of these precursors using glutamine, glutamate or NH_4 as amine group donors.

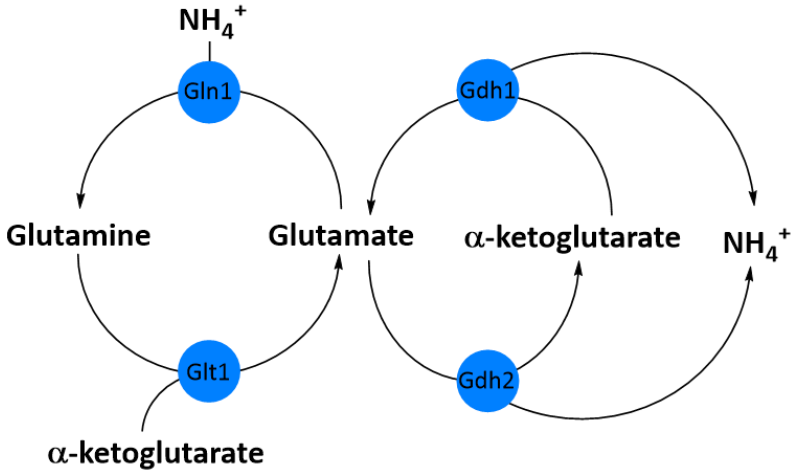

Figure 3. Central nitrogen metabolism. The interconversion of glutamine, glutamate, α-ketoglutarate and ammonium (NH_4) is considered the nitrogen metabolic hub. The amino group and ammonium ions obtained from the assimilation of the nitrogen source are recycled between these metabolites and other reactions. The enzymes involved are Glutamine synthetase (Gln1), Glutamate dehydrogenase (Gdh1), Glutamate synthase (Glt1) and Glutamate dehydrogenase (Gdh2).

4.2.3. Nitrogen regulation pathways

Since nitrogen in an essential nutrient for yeast, the mechanisms of *de novo* synthesis, metabolic conversions and transport of amino acids are thoroughly regulated at different levels (Bianchi et al., 2019). The regulation can be at the transcriptional level, where the expression of genes encoding amino acid transporters and proteins involved in nitrogen metabolism is controlled depending on the nutritional status of the cell and the availability of substrates. The regulation can also be at the protein level, which mainly consists of post-translational modifications and allosteric control. Finally, regulation takes place at the vesicle level by trafficking and endocytosis, where the transporters location and degradation are controlled. Although there are separate mechanisms, the different pathways are interconnected and work in association to regulate and control nitrogen metabolism. In this introductory section, we described in detail the nitrogen regulation pathways at gene expression level because these are more related to the response to different environmental conditions. These have also been reported to be directly involved in the regulation of flavour and aroma compounds production.

4.2.3.1. NCR

Nitrogen metabolism in *S. cerevisiae* is regulated by the Nitrogen Catabolite Repression (NCR) (Magasanik and Kaiser, 2002). This mechanism ensures the effective use of preferred nitrogen compounds when they are available, at the expense of poor nitrogen sources. This process is mediated by GATA transcription factors that are activators (Gln3, Gat1) or repressors (Dal80 and Gzf3) of the transcription of genes involved in nitrogen catabolism, nitrogen transport and degradation of proteins (Hofman-Bang, 1999). Ure2 is a negative regulator acting at allosteric level. The activity of the NCR has been used as referent to determine the nitrogen preference by yeast (Ljungdahl and Daignan-Fornier, 2012), although it depends on the yeast strain.

4.2.3.2. SPS-sensor

Another regulation process of nitrogen uptake in *S. cerevisiae* is the SPS-sensor system that controls the expression of specific nitrogen permeases in response to extracellular amino acid availability. The sensor complex, composed by Ssy1p-Ptr3p-Ssy5 (SPS), is localised in the plasma membrane to detect the presence of external amino acids (Forsberg and Ljungdahl, 2001). When the sensor recognizes amino acids, it catalyses the protein modification of the cytosolic transcription factors (Stp1 and Stp2) involved in induction of amino acid permeases. Then, the modified Stp1 and Stp2 are targeted to the nucleus, where they bind the permeases promoters to induce transcription (Ljungdahl, 2009).

4.2.3.3. GAAC

The general amino acid control (GAAC) is a pathway that responds to amino acid starvation by inducing the expression of genes involved in amino acid biosynthesis and some transporters. The regulation is mainly mediated by Gcn4 that is the transcription factor in charge of activation and/or repression of target nitrogen metabolism genes (Natarajan et al., 2001). The mechanism by which Gcn4 is induced to further regulate gene expression, is inhibition of translation initiation that involves four upstream open reading frames (uORFs) and activation of Gcn2 by uncharged tRNA (Hinnebusch, 2000).

5. Flavour and aroma in fermented beverage

The contribution of aroma compounds to fermented beverage come from the raw materials (varietal aromas), the fermentation (fermentative aromas), and the maturation processes (ageing aromas). The literature reports that the final concentrations of various odour-active components (OAC) are highly dependent on yeast during fermentation. The OAC originated from the secondary metabolism of yeast (metabolic intermediates or fermentation by-products) include volatile substances like

esters, higher alcohols, and carbonyl compounds. The presence of these compounds is important for the production of wine, beer, cider, sake, sherry, brandy, rum, whiskey and other fermented beverages (Querol and Fleet, 2006).

5.1. Volatile compounds

The volatile compounds related to desired flavours and aromas have been categorized in two main groups, higher alcohols and esters. The characteristics and biosynthetic pathways of the aroma compounds produced by yeast are described in this section.

Table 1. Most important aroma compounds present in fermented beverages.

Amino acid	Ketoacid	Higher alcohol	Aroma	Ester	Aroma	Acid
Valine	α-ketoisovalerate	Isobutanol	Spirits, fusel	Isobutyl acetate	Fruity	Isobutyrate
Isoleucine	α-ketomethylvalerate	Amyl alcohol	Marzipan	Amyl acetate	Banana	Methylvalerate
Leucine	α-ketoisocaproate	Isoamyl alcohol	Fruity, wine	Isoamyl acetate	Banana	Isovalerate
Methionine	α-keto-4-methylthiobutyrate	Methionol	Potato, candy	-	-	2-methylthiopropanoate
Phenylalanine	Phenylpyruvate	2-phenylethanol	Roses	2-phenylethyl acetate	Fruity, floral	2-phenylacetate

5.1.1. Higher alcohols

Higher alcohols, also called fusel alcohols, are secondary metabolites produced in alcoholic fermentation that positively contribute to beverage quality and aroma, when present at appropriate concentration. These are the most abundant organoleptic compounds produced in beer and wine, and at the proper concentration give sweet, fruity, and floral notes to the alcoholic beverage (Table 1). However, at very high concentrations these alcohols are toxic for the yeast cell and also negatively influence the aroma. Higher alcohols are produced during fermentation through the Ehrlich pathway (Hazelwood et al., 2008), which is a catabolic route allowing the

conversion of exogenous amino acids to higher alcohols through three main enzymatic steps illustrated in Figure 4. The Ehrlich pathway steps are explained as follows:

- Transamination: The reaction consists in the transfer of amino group from the amino acid to another molecule to form the respective α-ketoacid. There are four transaminases catalysing the reaction (Bat1, Bat2, Aro8 and Aro9) that use glutamate/α-ketoglutarate as donor/acceptor (Pires et al., 2014). An alternative to the transamination step to produce α-ketoacid is related to anabolism, through the production of these intermediates from carbohydrate metabolism (Suomalainen and Lehtonen, 1979). Ketoacid intermediates appear as a link between degradative and synthetic processes (Huergo and Dixon, 2015; Kerber and Fernando, 2010), as these compounds can originate from the sugar metabolism during wine fermentation (Crépin et al., 2017; Rollero et al., 2017).
- Decarboxylation: This is an irreversible step where the α-ketoacids generated from the transamination step lose a carbonyl group to form the respective aldehyde, releasing CO_2 (Dickinson et al., 1997). This reaction can be catalysed by five decarboxylases encoded by *PDC1, PDC5, PDC6,* and *ARO10* genes. The activity of these enzymes depends on the thiamine diphosphate (TPP) cofactor and just some of them are substrate specific.
- Reduction to higher alcohols: This is the last step of the Ehrlich pathway. Alcohol dehydrogenases (ADH) convert the fusel aldehyde into their respective fusel alcohol (Dickinson et al., 2003). The reaction uses NAD^+ as cofactor.

Besides the reduction step, there is a parallel oxidation of aldehydes to form fusel acids that plays a minor role in yeast. The balance of these two reactions depends on the fermentation conditions. In fermentations done with *S. cerevisiae* in anaerobic conditions, the production of fusel alcohols is 90% higher than fusel acids (Hazelwood et al., 2008), although

it mainly depends on the redox status of the cell. At the end of the pathway, the cells release the fusel alcohols to the culture medium through simple passive diffusion (Lipinski et al., 2001) or with the action of an ATP-dependant exporter in the case of the fusel acids (Hazelwood et al., 2006).

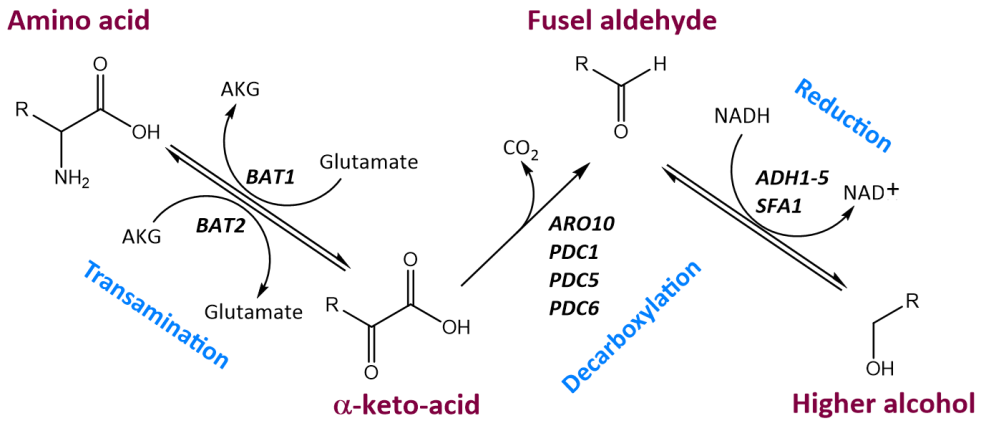

Figure 4. The Ehrlich pathway. The aromatic and branched chain amino acids are catabolised through the Ehrlich pathway. First, the amino group of the amino acid is transferred to α-ketoglutarate to form glutamate and the α-ketoacid. Then, the carboxyl group of the α-ketoacid is released as CO_2, generating an aldehyde. Finally, the aldehyde is reduced to higher alcohol with the addition of H^+ coming from the cofactor NADH. Genes are in bold. α-ketoglutarate (AKG). Radical group (R).

5.1.2. Esters

Esters are other group of volatile compounds that significantly contribute alcoholic beverages, defining the final aroma depending on the concentrations produced by yeast. Low amount of these elements is enough to generate pleasant odours but when the compounds are overproduced there is negative effect in the final aroma (Liu et al., 2004). There are three major groups of esters important in aroma production by yeast:

- Acetate esters: In yeast cells, acetate esters are present in higher concentrations than other esters. The formation consists in the transfer of an acetate group from acetyl-CoA to ethanol or a higher alcohol, therefore, the availability of acetyl-CoA is important for the

production of acetate esters. The enzymes involved in this reaction are alcohol acetyltransferases (AATases), encoded by the paralogues *ATF1* and *ATF2* (Zhang et al., 2013). In addition, the Eat1 enzyme is also responsible for ethyl acetate synthesis in *S. cerevisiae* (Kruis et al. 2017).

- Medium-chain fatty acid (MCFA) ethyl esters: The production of these esters by yeasts is low compared to the acetate esters. Saerens *et al.* (2006) determined that the MCFA ethyl esters are the result of a condensation between acyl-CoA and ethanol. The reaction is catalysed by acyl-CoA/ethanol O-acyltransferases (AEATases) that are encoded by *EHT1* and *EEB1*, with *EEB1* the most relevant (Saerens et al., 2006).
- Branched acid ethyl esters: These are also referred as substituted ethyl esters and comprise the third group of ethyl esters identified in fermented beverage (Díaz-Maroto et al., 2005). These esters are produced in small amounts in *S. cerevisiae,* but production increases during the aging stages of alcoholic beverage. The substituted ethyl esters containing an alkylated and/or hydroxylated chain, result from the chemical esterification of ethanol with the fusel/branched acids produced by yeast. Although the enzymes controlling the esterification step have not been recognised, it was recently found that *MGL2* and *YJU3*, encoding mono-acyl lipases, potentially have a role on substituted ethyl esters metabolism (Marullo et al., 2021).

5.2. Regulation of aroma metabolism

The process of volatile metabolite formation involves transporters, catabolic enzymes, and transcription factors as aroma compound production mainly depends on the availability of precursors and the regulation of the process. Therefore, here we describe the related enzymes and regulation systems.

As the transamination step of the Ehrlich pathway is the start point of the

fusel alcohols formation, its regulation significantly affects the quantity of volatile compounds produced. The four genes that encode the enzymes involved in this reaction are *ARO8, ARO9, BAT1* and *BAT2.* Having said that, all the molecules in contact with these genes and their respective enzymes are studied as regulators of this pathway. Aro80 is a specific regulator that induces the transcription of *ARO9* and *ARO10* (encoding decarboxylases) by binding their promoters in the presence of aromatic amino acids (Iraqui et al., 1999). This protein works together with Gat1 and Gln3 activators involved in the NCR system to induce transcription (Lee and Hahn, 2013).

Acetate ester production depends on activity of the anabolic enzymes (AATases) encoded by *ATF1* and *ATF2*, which have different transcription levels depending on the strain and the environmental conditions. A direct correlation has been found between an increase of acetate ester formation and the increased expression of *ATF1,2* by *S. cerevisiae* as a consequence of nitrogen addition (Seguinot et al., 2018). Gamero et al. (2014) compared the expression levels of genes (*ATF1* and *ATF2* included) involved in formation of aroma compounds in three yeast species at low temperatures, and notable differences were observed in the gene expression and the final aroma profile. These results suggest that the yeast species have different regulation system and that these genes are crucial in the aroma production. On the other hand, catabolic enzymes (esterases) cleave the esters or avoid the formation of ester bonds. The study done by Ma et al. (2011) proved that the insertion of a C-terminus in the active site of an esterase, makes it non-functional to breakdown the formed esters and therefore the amount of esters increases. Therefore, the active esterases available in the yeast cells are important regulators to maintain the acetate esters balance. Although the reverse activity of esterases produces ethyl acetate from acetic acid and ethanol, the high concentrations of ethyl acetate in the yeast cells is attributed to the activity of Eat1 family enzymes (Kruis et al., 2017). Moreover, a study on the production of acetate esters and fusel alcohols by *S. kudriavzevii, S. uvarum* and *S. cerevisiae* on phenylalanine, reported that

S. uvarum showed high production of the respective acetate ester while *S. kudriavzevii* had more production of 2-phenylethanol, explained by the fact that the genes involved in amino acid metabolism were up-regulated in *S. uvarum* compared to *S. kudriavzevii* (Stribny *et al.* 2015). In contrast, Gamero et al. (2014) reported that *S. kudriavzevii* showed up-regulation of *ATF2* compared to *S. uvarum*, probably because of the high concentration of higher alcohols as precursors of esterification.

5.3. Factors affecting aroma production and fermentation

As it was mentioned before, external factors that significantly influence fermentation largely do so by influencing the yeast response to the environmental conditions. The most important factors in alcoholic fermentation are the temperature, sugar content, nitrogen source, oxygen availability and pH. The way in which these factors impact the yeast metabolism is mainly on the activity and stability of proteins, and on gene expression regulated by nutrient availability. The modulation of external parameters allows the obtention of the desired quality of product. Nonetheless, focusing on the sensory profile of alcoholic beverages, several studies demonstrated that the nitrogen source and the temperature in relation to the yeast strain are the conditions that most significantly impact the production of flavour and aroma compounds.

5.3.1. Nitrogen source

The substrates used in fermentations for alcoholic beverage are rich in carbon sources but often limited in nitrogen source. The nitrogen compounds utilised by yeast to grow are present at variable levels in natural musts and worts. Studies showed that yeasts require a minimal concentration of 140 mgN/L to complete sugar fermentation, however, this value depends on sugar concentration. As an alternative to conditions of nitrogen limitation, nitrogen can be added only in form of ammonium or yeast derivative, but it is not a systematic practice. The amount of nitrogen

present in fermentation is carefully controlled as it significantly affects the yeast activity (Varela et al., 2004). In alcoholic beverage production, the YAN, composed by amino acids, ammonium ions and small peptides (Ugliano et al., 2007), can vary depending on the fermentation media and the different obtention processes and viticultural practices (Drumonde-Neves et al., 2021; Pretorius, 2020). The effect of nitrogen content during alcoholic fermentation in oenological conditions has been studied by many researchers using synthetic must (MS) that mimics the composition of natural grape juice. The MS formula used in this study was obtained from Bely et al., (1990) who standardized the MS analysing 40 musts corresponding to 25 different grape varieties from several French regions and different pre-fermentative techniques. The advantage of using MS is that it was adjusted to represent several grape varieties, plus the exact concentration of each component is known, allowing total control over the fermentation parameters.

The YAN in fermentation is considered to be well correlated with the fermentation performance and the biomass production of *S. cerevisiae* (Varela et al., 2004). Furthermore, Rollero *et al.* (2018) studied the fermentation performance, nitrogen uptake and volatile compound production in non-traditional yeasts, and reported that the yeasts with efficient nitrogen uptake produced more biomass and had the highest fermentation rates. Moreover, nitrogen has a major effect in the biosynthesis of flavour and aroma compounds. The type and amount of free amino acids assimilated by yeast lead to the formation of higher alcohols through the Ehrlich pathway (Hazelwood et al., 2008), which also leads to the conversion to esters. Valine, leucine, isoleucine and phenylalanine are the main amino acids that increase the formation of their respective higher alcohol when added at high concentration (Procopio et al., 2013). Additionally, other amino acids such as glutamine, which is one of the most common amino acids in the grape must, are not converted into higher alcohols via Ehrlich pathway in yeast but still influence the volatile

compounds production by affecting the cell growth. The type and level of nitrogen does not affect the organoleptic properties in the same way for all yeasts, thus, the aroma compounds produced during fermentation depend on the yeast strain genotype and metabolic response. Stribny *et al.* (2015) for instance, evaluated the higher alcohols and acetate esters produced by three different *Saccharomyces* species in different nitrogen sources but with the same fermentation conditions. Although the use of a specific type of nitrogen source increases or decreases the formation of specific compounds, the difference in the results is attributed to the amino acid metabolism and the regulatory system of each species. In the presence of the same amount of phenylalanine, for example, *S. uvarum* produced high amounts of 2-phenylethyl acetate, while *S. kudriavzevii* produced more 2-phenylethanol.

A considerable amount of literature has been published on the topic of nitrogen assimilation in *S. cerevisiae* and its relation to aroma compounds production (Crépin et al., 2017; Gobert et al., 2019). Yet, much less is known about this relation in other yeasts. The order of uptake of free amino acids for instance, is strain dependent among non-conventional wine yeasts and the highest consumption of nitrogen was not necessarily correlated to highest production of aroma compounds (Rollero *et al.* 2018). The aroma production is related to the presence and activation of specific genes encoding catabolic and biosynthetic enzymes, therefore, determining the nitrogen preference is important because the growth rate impacts the gene expression levels and the general physiology of the cell (Boer et al., 2007).

5.3.2. Temperature

The temperature used for fermentation determine the yeast metabolism and the physical characteristics of the media. For the production of fermented beverages, the temperature used varies between 10°C to 30°C depending on the yeast. The temperature affects growth and the volatile compounds production in different ways. First, this factor is an essential

determinant of the yeast cell function. The reactions taking place in the cell can be optimal depending on the temperature tolerance of the strain, and this is linked to the regulation of specific genes and the enzymes activity. In a brewing *S. cerevisiae* strain for instance, increasing the temperature of fermentation promoted the expression of *BAP2*, a gene encoding an amino acid permease. This increased the availability of amino acids inside the cell and the formation of higher alcohols (Kodama *et al.* 2001). Likewise, Saerens et al., (2008) showed that, in beer fermentation, the expression of *ATF1* and *ATF2,* encoding for alcohol acetyltransferases (AATase), involved in formation of acetate esters, increased at higher temperatures. These studies suggest that the fusel alcohols formation can vary at different temperatures, also affecting the formation of esters, as the higher alcohols are their precursors. Secondly, the temperature influences ethanol production. At higher temperatures, yeast decreases the ability to convert glucose to ethanol and decreases its tolerance to this product (Hacking et al., 1984). However, ethanol production at high temperatures has advantages such as reducing cooling costs, decrease the likelihood of contamination and the possibility to remove ethanol continuously (Banat et al., 1998). This highlights the importance of non-conventional yeasts such as *K. marxianus,* which is thermotolerant and can be used for fermented beverage production and other industrial processes. Finally, the temperature indirectly affects the fermentation by altering the oxygen solubility in the medium. The dissolved oxygen has an impact on the yeast growth and determines the metabolic route for energy production (fermentation or respiration). In addition, the production of esters and other volatiles is reduced due to high oxygen availability. Fujiwara *et al.* (1999) reported that the dissolved oxygen is a negative regulator of esters synthesis by repressing *ATF1* gene with a hypoxic repressor complex. Beyond the biological effect of temperature, there is a physiochemical effect in fermentation leading to losses of volatile compounds by evaporation.

6. Approaches to evaluate yeast performance and aroma production

6.1. Growth kinetics and production of central carbon metabolites

Yeast growth is a result of the interaction of the yeast with its environment. The evaluation of the growth phases of yeast determines the response to the nitrogen source since it is a limiting nutrient. Therefore, growth kinetics evaluation is important to understand the aroma production in yeast, taking into account that some nitrogen compounds are precursors of aroma molecules (Fairbairn et al., 2017). The most-used approach to evaluate the nitrogen preference is evaluating the growth rate, the activation of nitrogen regulation pathways (Ljungdahl and Daignan-Fornier, 2012) and the order of consumption when nitrogen is provided as a mix (Crépin et al., 2012). Nevertheless, the optimum growth is not directly related to the high production of aroma volatiles, in fact, the highest aroma compounds formation in *S. cerevisiae* and *S. uvarum* was seen during the stationary phase when the yeast entered in a nitrogen starvation state (Minebois et al., 2020). Furthermore, Ljungdahl and Daignan-Fornier (2012), after a compilation of research on nitrogen assimilation by *S. cerevisiae*, established that asparagine, aspartate, glutamine and glutamate, which are amino acids assimilated through the central nitrogen metabolism, are preferred nitrogen compounds, as well as ammonium that is a rich inorganic source. Phenylalanine and valine were classified as intermediate nitrogen sources as they result in higher doubling times, but inactivity of the general amino acid control (GAAC) pathway. Lastly, methionine, isoleucine and leucine were classified as non-preferred amino acids because of the long generation times plus the activation of GAAC. The mentioned intermediate and non-preferred amino acids are important precursors of aroma compounds since their catabolism is done through the Ehrlich pathway, however, the first step of conversion generates α-ketoacids and derivatives, which can inhibit cell growth (Hazelwood et al., 2008).

Besides growth, other fermentation parameters are important to understand the yeast behaviour in particular nitrogen conditions and the

relation to the aroma profile. The evaluation of pools of central carbon metabolites during fermentation describe the yeast activity. Carbon intermediates such as organic acids and α-ketoacids generated from the CCM play a critical role for the aroma compounds production. According to Crépin et al., (2017), the major contribution of carbon skeletons for volatile compounds formation come from the CCM. Important central carbon metabolites such as glycerol, α-ketoglutarate, succinate, acetate, and pyruvate give evidence of the physiological state of the cell and impact the wine balance and quality (Jackson, 2014).

Finally, growth kinetics differ from fermentation kinetics, which are determined by the sugar consumption rate. The conversion of sugar is coupled to CO_2 production rate, so the measurement of released CO_2 gives information about the fermentation performance, including metrics such as lag phase, total fermentation time, and growth and stationary phases. In oenological conditions, yeast has the highest CO_2 production rate during the growth phase, and it slowly decreases until sugar exhaustion. Therefore, in wine fermentations, nitrogen is the limiting nutrient that determines the end of the growth phase by the complete depletion of nitrogen that impairs growth.

6.2. Gene expression profile

The methods to study the complexity of gene expression in response to environmental factors have developed over the last decades. Techniques such as qPCR and microarrays (Naurin et al., 2008) are conventional methods that have been used to analyse the expression level of specific candidate genes. Currently, with the rapid development of next generation sequencing and the progress of bioinformatic tools, a broad and accurate knowledge of the genome has been achieved. With this approach it was possible to then focus on the transcriptome sequencing (RNA-Seq). RNA-Seq is a whole transcriptome shotgun sequencing that uses next generation sequencing to describe the RNA content of an organism. The information obtained from

this method is RNA reads that are aligned onto the reference transcripts or are reconstructed as *de novo* assembly of transcripts. Moreover, to analyse the gene expression levels in the sample, the reads are quantified to obtain the transcript abundance. The main advantages of using RNA-Seq for gene expression patterns analysis, also being the reasons of using this approach in the present study are that first, the data is directly derived from functional genomic elements such as protein coding genes; second, it is possible to obtain both the sequence information and the measure of gene expression by sequencing; and third, it gives information about the transcribed genes and the RNA splice events included in all the metabolic pathways. On the other hand, this technique has some limitations that should be considered for an RNA-Seq experiment and potential improvement. The main limitation, also found in other gene expression techniques, is that when measuring the mRNA levels there is lack of information about the stability and turnover rates, so the mRNA quantity in the cell not always represents the protein abundance (Vogel et al., 2010). Additionally, the standard RNA-Seq does not incorporate strand specificity, thus the transcripts from overlapping genes encoded in different strands are hard to distinguish (Wolf, 2013). Most importantly, the genome sequence must be known to use as reference for mapping of the mRNA transcripts; this becomes an issue for organisms that lack the genomic reference sequence, or it is fragmented and altered. Nevertheless, 'assembly first' methods have been developed in the last decade using the RNA-Seq reads to directly assemble transcripts for *de novo* transcriptome assembly. One of those methods for *de novo* reconstruction of transcriptomes is Trinity, which consists of three sequential software modules (Inchworm, Chrysalis, and Butterfly) to process large volumes of RNA-Seq reads (Grabherr et al., 2013). Then, the assembled transcriptome can be used as reference.

6.3. Metabolome profiling

In order to modulate the aroma profile of alcoholic beverage, the metabolome of yeast strains used for fermentation should be known. The

metabolome refers to all the primary and secondary metabolites produced by the cell, including intermediate and the end products. The study of the metabolites produced by yeast during alcoholic fermentation can be done through separation and detection methods. The metabolites detected in the liquid phases in wine fermentations that are also used to describe the fermentation profile in yeast are sugars, ethanol, glycerol, pyruvate, acetate, succinate, malate, citrate, and α-ketoglutarate. The approach used for the identification and quantification of compounds dissolved in a liquid sample is high performance liquid chromatography (HPLC), invented in the 1960's (Karger, 1997). Moreover, chromatographic techniques are used to detect the volatile metabolites. Alves *et al.* (2015) established the volatile metabolome of commercial and wild *S. cerevisiae* strains used in wine production, identifying 257 volatile metabolites that were distributed over different chemical families. The most commonly used methodology for volatile metabolome is headspace solid phase micro extraction (HS-SPME) combined with two-dimensional gas chromatography coupled to mass spectrometry (GC×GC-MS). The HS-SPME uses a fused silica fibre coated with polymeric organic liquid which extracts and concentrates the volatile molecules to be transferred to the analytical instrument (Zhang and Pawliszyn, 1993). Following this extraction, the separation, identification and quantification technique used for volatile compounds is gas chromatography coupled to mass spectrometry (GC-MS), which consists of two parts: The GC that separates the molecules through a capillary column depending on the chemical properties and the affinity of the molecule to the phase, and the MS that ionizes and detects the molecules depending on the mass-to-charge ratio. The combination of these methods reduces the possibility of error in the molecule detection.

The integration of metabolome profiling and the transcriptome analysis allows understanding of cellular biology and physiological state of an organism in particular conditions. Previous studies have used integration of omics strategies to explore the molecular mechanisms causing specific

behaviours in yeast strains (Kresnowati et al., 2006; Mendes et al., 2017). Especially the analysis of aroma production in fermented beverages have revealed strong relationships between the variations at gene expression and metabolite production levels.

7. Aim of the study

The project focuses on how the environmental factors, interact with the genotype of novel yeasts to modulate the flavour and aroma production. The strain genotype influences the nitrogen assimilation and the fermentation performance, therefore, we focused on the nitrogen metabolism of yeast. The yeast response to a range of nitrogen compounds evaluated in this study have been previously described and classified in *S. cerevisiae,* however, much uncertainty still exists about these nitrogen compounds in *S. uvarum*. The volatile metabolome and gene expression data at different nitrogen conditions give valuable understanding of the yeast genetic response to these environmental conditions, and also the influence in pathways of aroma compounds. The outcomes of this research provide knowledge that leads to the development of novel fermented or distilled beverages with improved flavour and aroma characteristics. *K. marxianus* is the other non-traditional yeast used for this study. This species was chosen as a representative of an ancestral yeast that did not go through the whole genome duplication. Then, the comparison of molecular mechanisms of this yeast with *S. cerevisiae* provides knowledge on genome evolution and the mechanisms acquired by *S. cerevisiae* to perform as the number one domesticated yeast used for fermentation.

This doctoral thesis was part of the YEASTDOC programme (funded by the European Union H2020 Marie Skłodowska-Curie actions Agreement No 764927) which main objective was to maximize the potential of *Saccharomyces* and *non-Saccharomyces* yeasts in the beverage and white biotechnology sectors. In this context, for this thesis investigation, genomic, transcriptomic and metabolomic approaches were used to achieve the

following overall and specific objectives:

Objective 1. To understand how growth and metabolome of *S. uvarum* is strongly influenced by nitrogen assimilation in wine fermentations.

Based on the reported oenological differences between *S. uvarum* and *S. cerevisiae*, we focused efforts on comparing these yeasts in wine fermentations, variating the nitrogen source since nitrogen has an impact on yeast growth, fermentation and production of flavour and aroma. This was done with the purpose of understanding *S. uvarum* behaviour in relation to the nitrogen source and explain the unique attributes of this yeast.

Specific objectives

- Determine nitrogen preference of *S. uvarum* in terms of efficiency to support growth under oenological conditions.
- Investigate the impact of the nitrogen source on fermentation performance, production of central carbon metabolites and volatile compounds during wine fermentation with *S. uvarum*.
- Explain the distinctive phenotypic traits of this species by integrated analysis of nitrogen catabolism and the metabolome.

Results and discussion presented in Chapter 1.

Objective 2. To explore the transcriptional landscape of *S. uvarum* when growing using preferred and non-preferred nitrogen sources.

As it is known that growth and aroma profile of *S. uvarum* differ from *S. cerevisiae,* it follows that there must be differences in nitrogen metabolism. We decided to establish a base-line to understand *S. uvarum* nitrogen metabolism in relation to its attractive aroma profile by studying the transcriptome when growing on different nitrogen sources, namely

ammonium and amino acids that were either preferred or non-preferred nitrogen sources.

Specific objectives

- Explore nitrogen metabolism of *S. uvarum* grown in ammonium, asparagine, phenylalanine and methionine.
- Determine the effects of the nitrogen source on fermentation and aroma profile.
- Compare gene expression profile of *S. uvarum* to nitrogen metabolism of *S. cerevisiae* previously described in literature.
- Find the association of transcriptome and metabolome in *S. uvarum* in relation to the nitrogen source.

Results and discussion presented in Chapter 2.

Objective 3. To investigate the evolution and role of the periplasmic asparaginase Asp3 in yeast.

Having identified asparagine metabolism as a key difference between *S. cerevisiae* and *S. uvarum*, and knowing that *ASP3* was reported to be present in *S. cerevisiae* but not *S. uvarum*, we wanted to explore this gene and its contribution in more detail.

Specific objectives

- Evaluate the distribution and evolutionary trajectory of *ASP3* in yeast.
- Determine whether the presence of *ASP3* confers an advantage for yeast growth and nitrogen assimilation.
- Investigate whether fermentations of natural matrices (apple juice) support the idea that *ASP3* confers a selective advantage for *Saccharomyces spp.* in fermentative environments.

Results and discussion presented in Chapter 3.

Objective 4. To investigate the transcriptional regulation of *BAT1*, which encodes the branched-chain aminotransferase, Bat1, in *Kluyveromyces marxianus.*

Biosynthesis and degradation of branched chain amino acids are important during alcoholic fermentations. In post-WGD yeasts like *S. cerevisiae*, different genes encode the mitochondrial and cytosolic forms of this enzyme required, respectively, for these processes. In pre-WGD yeasts like *Torulaspora* or *Kluyveromyces*, there is a single gene that must encode enzymes for both functions. We chose to investigate *BAT1 in* K. *marxianus* to gain an insight into the differences in pre and post-WGD yeasts as this is relevant for application and for understanding the evolutionary processes that shaped *S. cerevisiae* as the pre-eminent yeast for alcoholic fermentation.

Specific objectives

- Study how *K. marxianus BAT1* synthesise proteoforms with different sub-cellular localisation relying on an integrated in silico and experimental approach.
- Explore condition-specific use of alternative transcription start sites to produce two mRNA isoforms.
- Establish how the use of alternative transcription start sites is regulated by specific transcription factors.
- Compare the regulation mechanism in *K. marxianus* with available data of *S. cerevisiae*, to understand the development of sophisticated expression control mechanisms in post-WGD yeast.

Results and discussion presented in Chapter 4.

Chapter 1

The growth and metabolome of *Saccharomyces uvarum* in wine fermentations is strongly influenced by the route of nitrogen assimilation

Angela Coral-Medina[1,2], John P. Morrissey[2,3], Carole Camarasa[1]

[1] SPO, Univ Montpellier, INRAE, Institut Agro, Montpellier, France; [2] School of Microbiology, University College Cork, Ireland; [3]Environmental Research Institute and SUSFERM Fermentation Science Centre, University College Cork, Ireland

Corresponding author: carole.camarasa@inrae.fr

ABSTRACT

Nitrogen is a critical nutrient in beverage fermentations, influencing fermentation performance and formation of compounds that affect organoleptic properties of the product. Traditionally, most commercial wine fermentations rely on *Saccharomyces cerevisiae* but the potential of alternative yeasts is increasingly recognised because of the possibility to deliver innovative products and process improvements. In this regard, *Saccharomyces uvarum* is an attractive non-traditional yeast that, while quite closely related to *S. cerevisiae*, displays a different fermentative and aromatic profile. Although *S. uvarum* is used in cider-making and in some winemaking, better knowledge of its physiology and metabolism is required if its full potential is to be realised. To address this gap, we performed a comparative analysis of the response of *S. uvarum* and *S. cerevisiae* to 13 different sources of nitrogen, assessing key parameters such as growth, fermentation performance, and the production of central carbon metabolites and aroma volatile compounds. We observed that the two species differ in the production of acetate, succinate, medium-chain fatty acids, phenylethanol, phenylethyl acetate and fusel/branched acids in ways that reflect different distribution of fluxes in the metabolic network. The integrated analysis revealed different patterns of yeast performance and activity linked to whether growth was on amino acids metabolised via the Ehrlich pathway or on amino acids and compounds assimilated through the central nitrogen core. This study highlights differences between the two yeasts and the importance that nitrogen metabolism can play in modulating the sensory profile of wine when using *S. uvarum* as the fermentative yeast.

INTRODUCTION

Nitrogen is one of the most important nutrients affecting wine fermentation. The composition and amount of nitrogen depend on the grape variety, viticultural management practices, soil, climate and degree of ripeness (Bell

and Henschke, 2005; Lafon-Lafourcade and Peynaud, 1959). This nutrient is present in grape juice as a complex mixture of nitrogen-containing compounds, only some of which - amino acids, ammonium and small peptides - are available to yeast. The total amount of nitrogen that can be accessed by yeast for growth is referred to as "Yeast Assimilable Nitrogen" (YAN) and its content in wine musts varies between 60 and 500 mg/L (Bely et al., 1990a; Ugliano et al., 2007). Two critical roles for YAN are described in *S. cerevisiae*, the quintessential yeast for producing alcoholic beverages. First, the nitrogen content of grape juice has been reported as the main factor limiting yeast growth during wine fermentation (Varela et al., 2004), with a direct impact on yeast fermentative activity and consequently on the fermentation kinetics (Bely et al., 1990b; Sablayrolles et al., 1996). It is generally accepted that YAN concentration below 150 mg N/L affects yeast metabolism and causes stuck or sluggish fermentations (Bell and Henschke, 2005; Blateryron and Sablayrolles, 2001; Sablayrolles et al., 1996), which are major issues in the wine industry. The presence of residual sugars associated with stuck fermentation is not desirable in many wine styles and becomes a risk for development of spoilage microorganisms. Second, studies assessing the influence of the quantity and quality of the nitrogen source on yeast metabolism revealed an effect on both the formation of central carbon metabolites, including glycerol, (Albers et al., 1996), and on the production of volatile compounds, which together affect mouthfeel, flavour and aroma (Gobert et al., 2019). In particular, it was reported that levels of YAN has a great impact on the formation of higher alcohols and branched acids (Rollero et al., 2017, 2015). To a certain extent this is because the branched and aromatic amino acids, which are catabolised through the Ehrlich pathway (Hazelwood et al., 2008), are precursors of volatile compounds in *S. cerevisiae* (Carrau et al., 2008; Fairbairn et al., 2017). In an indication of complexity, however, a quantitative analysis of nitrogen metabolism in *S. cerevisiae* demonstrated that direct catabolism of consumed amino acids, because of their low content in grape juice, were not major contributors to

the production of aroma compounds during wine fermentation (Crépin et al., 2017; Rollero et al., 2017).

When considering the production of volatile compounds, it is important to recognise that nitrogen catabolic and biosynthetic pathways function in parallel, with the balance between these coordinated by regulatory processes to generate an appropriate response to nitrogen availability (Ljungdahl and Daignan-Fornier, 2012; Zaman et al., 2008). The α-ketoacids originated from the Ehrlich pathway and the central carbon metabolism (CCM) are significant as they are precursors of both proteinogenic amino acids and aromatic/branched higher alcohols and acids. They play a key role in the nitrogen network behaving as metabolic nodes, around which the flux distribution is regulated depending on anabolic requirements and nitrogen availability. Furthermore, higher alcohols and branched acids are precursors for the synthesis of esters, compounds with a positive impact on the sensory quality of wine (fruity or floral notes). The formation of acetate esters result from the esterification of higher alcohols with acetyl-CoA by the acetyltransferases Atf1 and Atf2 (Lilly et al., 2006; Verstrepen et al., 2003), and branched ethyl esters from branched acids with ethanol by Eeb1 and Eht1 (Saerens et al., 2006). Other volatile compounds that contribute to wine aroma are the medium-chain fatty acids (MCFA) ethyl esters that result from the condensation of acyl-CoA and ethanol (Saerens et al., 2006) and possibly through a mono-acyl glycerol lipase (Marullo et al., 2021). These substances are related to lipid and acetyl-CoA metabolism, therefore, the fatty acids content of grape juice impacts the production of ethyl esters (Liu et al., 2019; Saerens et al., 2008). In addition, however, a slight impact of the nitrogen source on their production has also been reported (Barbosa et al., 2009; Seguinot et al., 2018).

The nitrogen sources that compose YAN are transported and assimilated by a wide range of permeases and catabolic pathways, supporting yeast activity in different manners (Bianchi et al., 2019; Zhang et al., 2018). Several different classifications that refer to distinct physiological mechanisms have

been proposed to assess the efficiency of these nutrients to support yeast activity. First, nitrogen preference based on the efficiency to support growth has been reported in *S. cerevisiae* where the amino acids were classified as not efficient (Lys, His and Gly), poorly efficient (Val, Phe, Leu, Ile, Met, Tyr, Thr, Trp and Pro) and highly efficient (Asn, Asp, Gln, Glu, Ser, Ala, Arg and NH_4) to sustain growth (Fairbairn et al., 2017; Godard et al., 2007; Watson, 1976). This classification is related to the activity through amino acid degradation pathways fulfilling anabolic requirements. Second, the efficiency to maintain high metabolic activity during the stationary phase of fermentation has been considered to determine the yeast nitrogen preference. This concept relies on the amount of nitrogen to be added during the stationary phase of wine fermentation to maintain a high metabolic activity, protein turnover and cellular maintenance (Brice et al., 2014; Manginot et al., 1998, 1997). Finally, the order of consumption of nitrogen sources provided as a mixture of ammonium and amino acids has been studied in wine context (Crépin et al., 2012). The differentiation between first consumed (Lys), early consumed (Asp, Thr, Glu, Leu, His, Met, Ile, Ser, Gln, and Phe), and late consumed (NH_4, Val, Arg, Ala, Trp, and Tyr) compounds is mainly associated to the regulation of permeases activity through NCR and SPS-sensor systems and the kinetic characteristics of transporters (Hofman-Bang, 1999; Ljungdahl, 2009).

In recent years, particular attention has been paid to so-called non-conventional or non-traditional yeasts as they offer outstanding alternatives for the innovation of fermented products and the improvement of processes (Drumonde-Neves et al., 2021; Fleet, 2008; Jolly et al., 2022; Pretorius, 2020). *Saccharomyces uvarum,* a close relative of *S. cerevisiae,* is one of the non-traditional yeasts associated with low ethanol production and strong aromatic intensity because of the high production of acetate esters (Stribny et al., 2016). *S. uvarum* is also a cryotolerant yeast meaning that it displays a good fermentation performance at low temperatures (12°C to 20°C) (Almeida et al., 2014; Zhang et al., 2015). These interesting traits make this

species very attractive for cider (González Flores et al., 2017) and wine production. In fact, some strains have already been commercialized and hybrids have been generated for winemaking and the brewing industry (Gamero et al., 2013; Gibson et al., 2017; Piatkowska et al., 2013). To date, information on *S. uvarum* metabolism has mainly focused on central carbon pathways, showing a high capacity of this species to produce glycerol and succinate, as well as an interesting system of acetate production-consumption to maintain the cryotolerant metabolism (Henriques et al., 2021; Minebois et al., 2020a). The characteristics of nitrogen metabolism and its relationship with the formation of volatile compounds are not well-documented in this species, with only one study reporting a moderate impact of nitrogen availability on fermentative capacities (Su et al., 2019). Nevertheless, such knowledge is essential for the managed use of *S. uvarum* as a credible alternative to *S. cerevisiae* for the modulation of the aroma profile of wine.

This study endeavours to provide a comprehensive picture of the impact of nitrogen sources on *S. uvarum* behaviour during fermentation and to explore the metabolic specificities of this yeast. To this end, the nitrogen preference of *S. uvarum* in terms of efficiency to support growth under oenological conditions was first determined. Then, the impact of the nitrogen source on fermentation performance, production of central carbon metabolites and volatile compounds during wine fermentation was characterised. The integrated analysis of the data set provided new insights on the relationships between the catabolism of nitrogen substrates and the metabolome in *S. uvarum* and provides some explanations for the distinctive phenotypic traits of this species. This research provides crucial fundamental knowledge that will facilitate informed use of *S. uvarum* to modulate the sensory profile of wine.

MATERIAL AND METHODS

Strains and pre-cultures

The strains used in this study are listed in Table 1. The strains were reactivated in YPD broth (glucose 20 g/L, yeast extract 10 g/L, peptone 20 g/L). The pre-cultures were grown in YPD at 28°C for 16 hours, then centrifuged 5 minutes at 4500 rpm. The pellet was suspended in Yeast Nitrogen Base (YNB) media (glucose 20 g/L) without ammonium and amino acids for 4 hours at 28°C to exhaust the nitrogen reserves. The cells were washed with 0.9% NaCl saline solution to remove all nitrogen residues and then resuspended in the test media without the nitrogen source. The cell suspension was further used to inoculate fermentations.

Micro-fermentations in minimal media

The micro-fermentations were done in microplates with 7 strains (Table 1). The minimal media (MM) components were glucose 20 g/L, KH_2PO_4 3 g/L, $MgSO_4.7H_2O$ 0.5 g/L, vitamin mix, and trace elements adapted from Verduyn media (Verduyn et al., 1992). Nitrogen was provided in excess, at the same concentration (1059 mgN/L) but in 10 different forms, ammonium or amino acids as sole nitrogen sources (Table 2). For the microplate fermentations, 20 µL of pre-culture suspension at ~0.5 A_{600} was added to 180 µL of fresh medium (~0.05 A_{600}) in each well. This experiment was done with three biological replicates of each condition using one blank per medium. The 96 flat wells microplate was incubated in the microplate reader CLARIOStar® Plus (BMG LABTECH, Germany) at 20°C. The absorbance at 600 nm was measured for 60 cycles of 1 hour (24 flashes/cycle) with continual double orbital shaking (500 rpm) between measurements.

Alcoholic fermentations in grape must

Fermentations in synthetic and natural grape must were conducted with *S. uvarum* MTF3098 and *S. cerevisiae* EC1118. Synthetic grape must (MS) was used to mimic the composition of grape juice and to allow varying the

nitrogen source, and natural musts which have a mixture of nitrogen sources were also used. The MS was prepared following the composition of Bely et al., (1990) with some adjustments. The base MS contained glucose 100 g/L, fructose 100 g/L, malic acid 6 g/L, citric acid 6 g/L, KH_2PO_4 0.75 g/L, K_2SO_4 0.5 g/L, $MgSO_4.7H_2O$ 0.25 g/L, $CaCl_2.2H_2O$ 0.155 g/L, NaCl 0.2 g/L, phytosterols 5 mg/L and trace elements and vitamins. The assimilable nitrogen was provided at 200 mgN/L as 11 different forms listed in Table 2. The pH of the MS was set to 3.3 with NaOH. The two natural white grape musts were Chardonnay (pH 3.78, 210 g/L sugar, 200 mgN/L) and Maccabeu (pH 3.5, 235 g/L sugar, 104 mgN/L) provided by the experimental unit INRAE Pech Rouge. The MS and natural musts were pasteurised for 20 minutes and then oxygenated by bubbling air for 30 minutes before inoculation.

Batch fermentations were performed in 330 mL fermenters containing 250 mL of medium, equipped with fermentation locks to avoid the entry of oxygen and allow CO_2 release. The initial population in each fermenter after inoculation was $5x10^5$ cells/mL (corresponding to A_{600} ~0.04 for *S. uvarum* and A_{600} ~0.05 for *S. cerevisiae*). The fermentations were carried out in biological triplicates at 20°C with continuous magnetic stirring (230 rpm) with an automated robotic system (PhenOFerm LabServices, Breda, Netherlands) capable of moving the fermenter from its location on the stirring plate to a precision balance (Duc et al., 2020). The fermentation progress was monitored by CO_2 (g/L) release, weighing the fermenters every hour to track weight and a custom-developed Labview application automatically calculated the amount of CO_2 released from the weight loss and the CO_2 production rate (g/L/h), which were determined based on polynomial smoothing (Sablayrolles et al., 1987). Samples for metabolome analysis were collected at 60 g/L of CO_2 produced and at the end of fermentation (CO_2 production rate lower than 0.05 g/L/h) by centrifuging 25 mL of medium (3500 g, 5 minutes, 4°C) to remove cells and storing the supernatant at -20°C.

Analytical and statistical methods

The yeast population at different fermentation times was quantified with a Coulter counter (BECKMAN®) and by measuring optical density (A_{600}) with a spectrophotometer. Growth kinetics data was analysed using R Studio software, version 1.3.1093 (RStudio Team, 2020). The area under the curve (AUC) was calculated with GrowthCurver package (Sprouffske and Wagner, 2016) as a measure of growth kinetics comprising the lag phase length, growth rate, final time and maximum biomass produced. The AUC metric has been used in previous studies comparing yeast growth depending on nitrogen source (Su et al., 2020). The boxplots, growth curves and statistical tests for growth analysis and metabolite production were made using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California, USA).

The methods for measuring metabolites and volatiles during wine fermentations were as previously described (Rollero et al., 2015). Metabolites such as glucose, fructose, ethanol, glycerol and organic acids were determined by High-Performance Liquid Chromatography (HPLC) on a Phenomenex Rezex ROA column (HPLC HP1100 Infinity, Agilent Technologies). The samples treatment for HPLC consisted of dilution of 200 µL of sample in H_2SO_4 0,005N solution, followed by purification with centrifugation in the case of MS and filtration for natural musts. From the same samples, the measurement of volatile compounds was performed by gas chromatography/mass spectrometry (GC-MS) with a Hewlett Packard 6890 gas chromatograph (Agilent Technologies, Santa Clara, California, USA) equipped with a CTC Combi PAL Autosampler AOC-5000 (Shimadzu, Columbia, USA), coupled to a HP 5973 mass spectrometry detector (HP, now Agilent Technologies, Santa Clara, California, USA). The liquid extraction of 5 mL supernatant was done with dichloromethane CH_2Cl_2; the organic phase was recovered, dried, and concentrated with nitrogen gas before injection. The measured aromatic compounds were higher alcohols, esters and fatty acids. The metabolome data was graphed using GraphPad Prism v8.0.2. and

two-way ANOVA was performed for heatmaps with correction for multiple comparisons using Tukey's test, where a p-value <0.05 was considered significant. The raw data for both growth and the metabolome in different conditions are available on Zenodo DOI:10.5281/zenodo.6627770.

To get an overview of correlation of the nitrogen source and metabolites produced, a principal component analysis (PCA) was performed for each group of volatile compounds using the R Studio software (Supplementary Material 1). Additionally, the complete analysis of two strains, 13 nitrogen sources and all metabolites was performed with XLSTAT software Version 2021.2.2.

Table 1. Strains used in this study

Strain	Environment	Geographical origin	Reference
S. uvarum MTF3098/BMV58	Wine	Valencia, Spain	Velluto BMV58™ from Lallemand Inc.
S. uvarum CBS395/CLIB251	Fruits: Juice of Ribes nigrum	The Netherlands	Pulvirenti et al., 2000
S. uvarum OS24/CBS7001/MCYC623	Insect Mesophylax adopersus	Avila, Spain	Kellis et al., 2003
S. uvarum OS472/A4	End of wine fermentation Sauvignon Blanc	Marlborough, New Zealand	Zhang et al., 2015
S. cerevisiae EC1118	Wine	Champagne, France	Novo et al., 2009
S. cerevisiae CEN.PK113-7D	Laboratory	-	Nijkamp et al., 2012
S. cerevisiae S288c	Laboratory	-	Mortimer and Johnston, 1986

Table 2. Nitrogen conditions tested in micro-fermentations and comparative synthetic wine must (MS) fermentations. The amount of assimilable nitrogen was 200 mgN/L in synthetic must (MS) and 1059.48 mgN/L in minimal media (MM); * g/L solubility limit of amino acid; † mix of amino acids as described by Bely et al., (1990).

Nitrogen condition	Nomenclature	Number of assimilated nitrogen atoms per molecule	Concentration of nitrogen compound	
			Micro-fermentations in MM (g/L)	Fermentations in MS (g/L)
Ammonium sulfate	NH_4	2	5	0.94
Methionine	Met	1	11.29	2.13
Phenylalanine	Phe	1	12.5	2.36
Asparagine	Asn	2	5	0.94
Aspartate	Asp	1	4.5 *	1.90
Valine	Val	1	8.87	1.67
Isoleucine	Ile	1	9.93	1.87
Leucine	Leu	1	9.93	1.87
Glutamine	Gln	2	5.53	1.04
Glutamate	Glu	1	7.5 *	2.10
MS 200 mgN/L	MS200	N/A	N/A	Ammonium chloride 0.216, mix of amino acids 6.16 mL/L †

RESULTS

Nitrogen preference in *S. cerevisiae* and *S. uvarum* species

To evaluate nitrogen preference, we monitored the growth of three *S. cerevisiae* strains and four *S. uvarum* wine strains (Table 1) during micro-fermentations conducted in MM varying the nitrogen source. The area under the curve (AUC) values, which provide an overall view of the efficiency of the nitrogen source to support growth, were compared between species (Figure 1A). Both species have the same growth capacity in most of the nitrogen sources, namely, good growth on ammonium, aspartate and glutamine, intermediate behaviour on phenylalanine, leucine, valine and glutamate, and poor growth on isoleucine. On the other hand, methionine and asparagine showed different efficiency to support growth between the two species. *S. uvarum* strains grew efficiently in methionine while *S. cerevisiae* strains had poor growth. In contrast, growth in asparagine was low for *S. uvarum* but very high for *S. cerevisiae*. However, the nitrogen preference can also be strain specific (Figure 1B): for example, *S. cerevisiae* CEN.PK113-7D showed a slower growth than the other *S. cerevisiae* strains in aspartate, while the lowest growth on isoleucine and methionine was observed with strains EC1118 and S288c, respectively. Regarding *S. uvarum,* strain CBS472 showed a different nitrogen preference than other *S. uvarum* strains, most notably a higher growth capacity on isoleucine than on leucine.

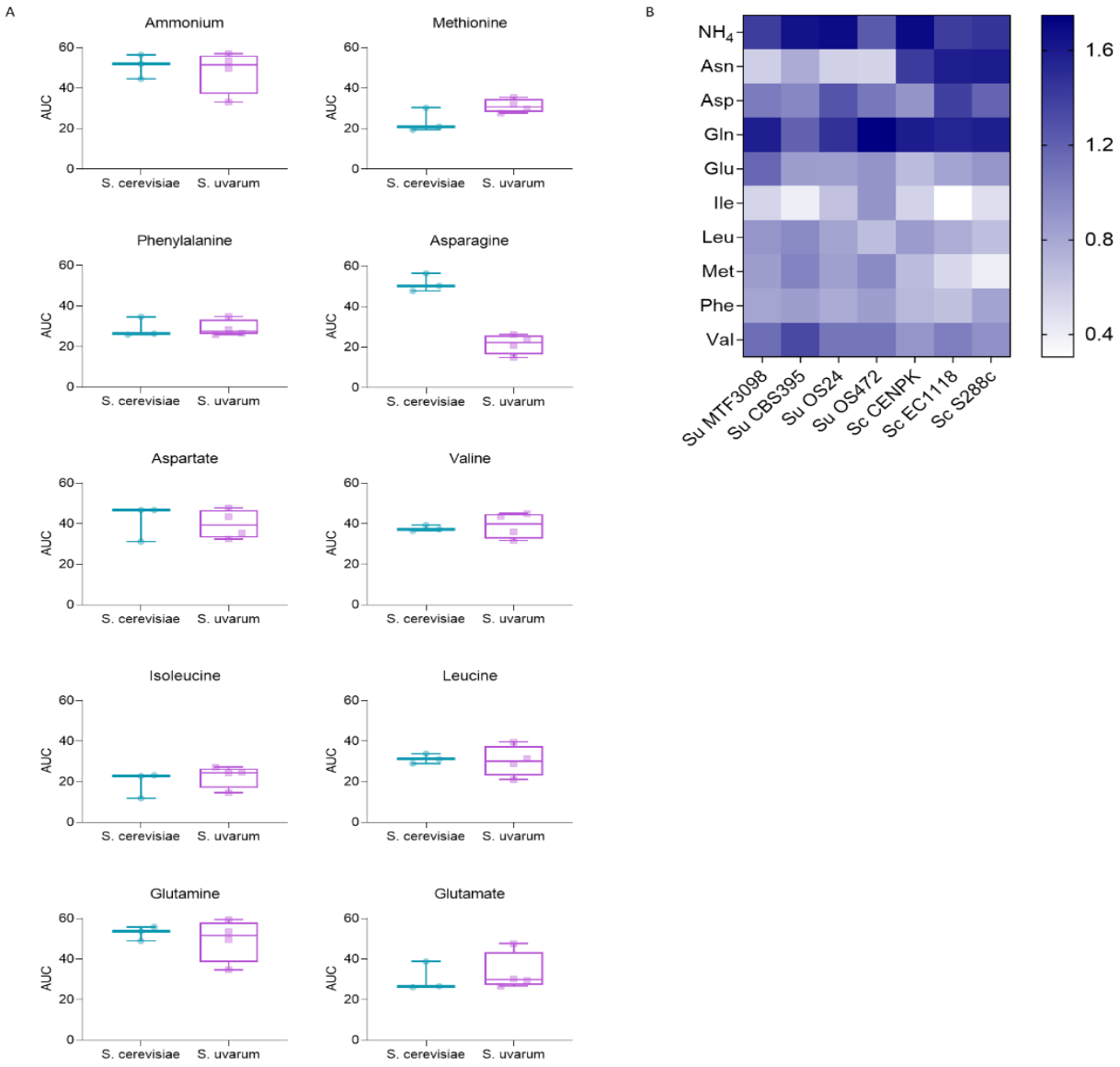

Figure 1. Growth efficiency in different nitrogen sources. Micro-fermentations were performed in minimal media supplemented with ammonium (NH_4), methionine (Met), phenylalanine (Phe), asparagine (Asn), aspartate (Asp), valine (Val), isoleucine (Ile), leucine (Leu), glutamine (Gln) and glutamate (Glu) as a sole source of nitrogen. Fermentations were at 20°C for 48 hours and growth determined using the area under the curve (AUC) method. (A) Area under the curve (AUC) of *S. cerevisiae* and *S. uvarum* species in different nitrogen sources. The evaluated strains were: *S. cerevisiae* (CEN.PK113-7D, EC1118, S288c) and *S. uvarum* (MTF3098, CBS395, OS24, OS472). (B) Growth of *S. uvarum* (Su) and *S. cerevisiae* (Sc) strains expressed in AUC. Values were normalized with the mean of all the strains for each nitrogen source. This allowed the classification of the strains according to their capacity to use a nitrogen compound. The highest AUC represents more efficient growth while low AUC indicate difficulty to assimilate the nitrogen source and sustain growth.

Effect of the nitrogen source on fermentation kinetics and profile

To further understand how the nitrogen source affects the fermentation profile in *S. uvarum*, the representative wine strains *S. uvarum* MTF3098 and *S. cerevisiae* EC1118 (used as reference) were grown in two natural musts and eleven synthetic grape juices with different nitrogen sources (Table 2). Sluggish profiles were observed during fermentations with *S. uvarum* but not with *S. cerevisiae* under the same conditions (Figure 2A). Overall, *S. cerevisiae* displayed higher fermentation performances than *S. uvarum.* This is illustrated with the considerably shorter time required to complete the fermentation for *S. cerevisiae,* where it ranged from 234 to 377 hours, compared to S. *uvarum,* where the range was between 401 and 477 hours depending on the nitrogen source (Figure 2B). In line with these observations, the maximum fermentation rate (Rmax), was in general lower in *S. uvarum* (0.51 g/L/h in asparagine to 0.75 g/L/h in leucine) than in *S. cerevisiae* (0.7 g/L/h in phenylalanine and 0.99 g/L/h in glutamine) (Figure 2C, Supplementary Table 1). The largest differences between the two strains were found in glutamine and asparagine fermentations, with 56% and 45% of variation, respectively. In methionine and isoleucine, *S. uvarum* had lower Rmax and fast decrease of fermentation rate that caused increase of total fermentation time. Surprisingly, *S. cerevisiae* displayed a longer lag phase than *S. uvarum* in most of nitrogen sources, except for asparagine. The lag phase of *S. uvarum* strongly depended on the nitrogen source, being higher than 50 hours in Ehrlich pathway amino acids and less than 50 hours in nitrogen compounds assimilated through the central nitrogen metabolism, except for asparagine (80 hours).

In addition, the two species differed in their capacity to deplete sugars. At the end of fermentation*, S. cerevisiae* exhausted sugars in most of the conditions, except from Maccabeu and phenylalanine. Conversely, *S. uvarum* depleted sugars only when isoleucine or leucine were used as the sole nitrogen source. Low amounts of residual sugars (<5 g/L), mainly in the form of fructose, were found during fermentations with ammonium, valine,

phenylalanine, and MS200, intermediate levels with glutamate and methionine, and high concentrations (>11 g/L) on glutamine, aspartate, and asparagine. Hence, the nitrogen source influences the capacity to consume sugars in *S. uvarum,* showing higher fermentation efficiency when the nitrogen source is provided as Ehrlich pathway amino acids, compared to amino acids directly incorporated through the central nitrogen metabolism.

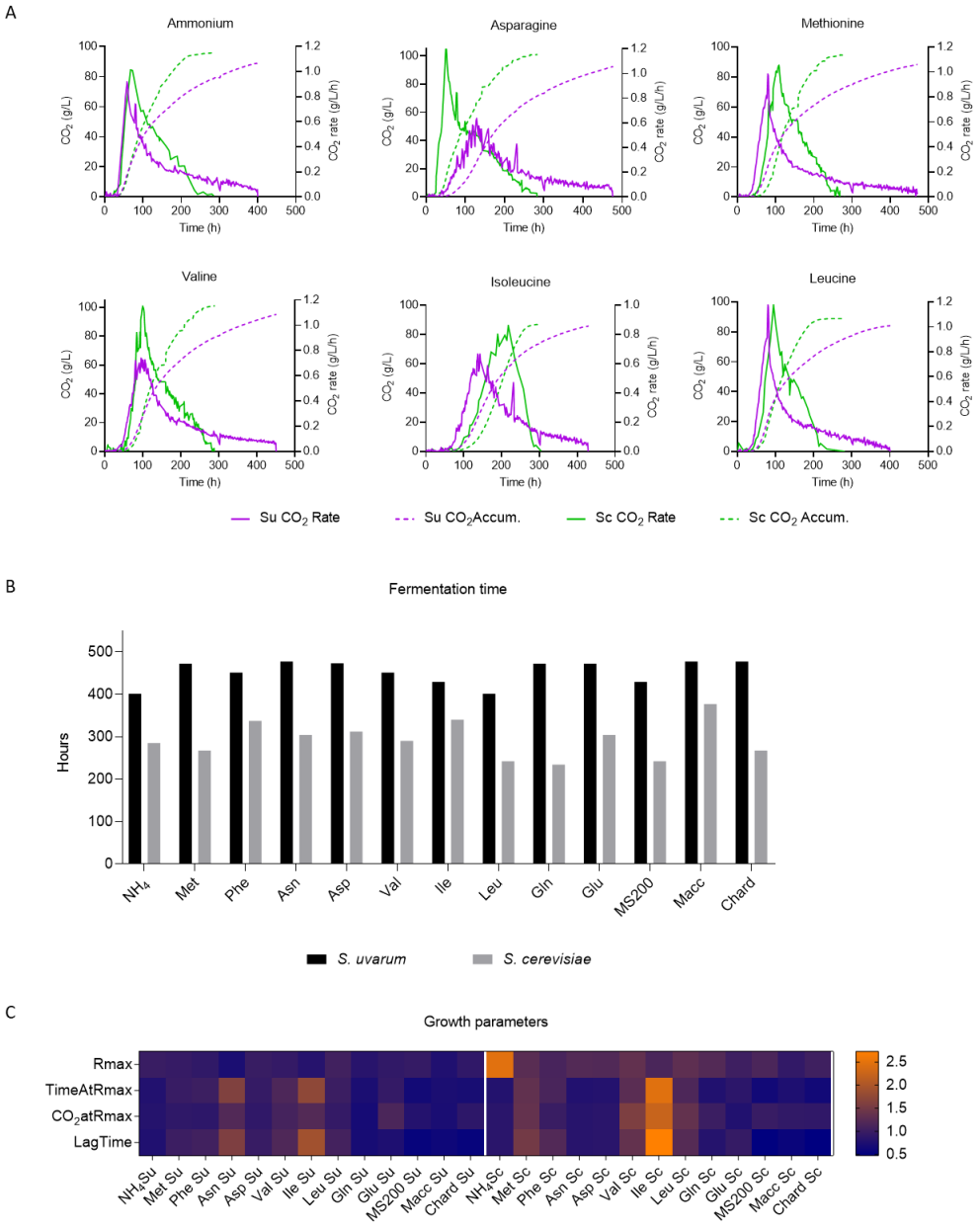

Figure 2. Growth kinetics of *S. uvarum* MTF3098 (Su) and *S. cerevisiae* EC1118 (Sc). Fermentations were conducted in synthetic must (MS) in 13 different nitrogen conditions, at 20°C until the CO_2 production rate was lower than 0.05 g/L/h. (A) Fermentation profile on ammonium, asparagine, methionine, valine, isoleucine and leucine. The accumulated CO_2 is expressed in g/L and the CO_2 production rate is expressed in g/L/h. (B) Fermentation

time expressed in hours in 13 different nitrogen conditions: ammonium (NH_4), methionine (Met), phenylalanine (Phe), asparagine (Asn), aspartate (Asp), valine (Val), isoleucine (Ile), leucine (Leu), glutamine (Gln), glutamate (Glu), MS200, Maccabeu (Macc) and Chardonnay (Chard). (C) Heatmap of the growth parameters of *S. cerevisiae* and *S. uvarum* depending on the nitrogen sources. Fermentation rate (Rmax), time passed until reaching Rmax (TimeAtRmax), CO_2 produced at the Rmax point (CO_2atRmax), duration of the lag phase (LagTime). The values were normalized with the mean of each parameter among the strains and all the conditions tested.

Central carbon metabolites during wine fermentation

The physiological behaviour of *S. uvarum* during wine fermentation with different nitrogen sources was investigated by comparing the profile of central carbon metabolites between the species. It is immediately apparent that the ethanol production by *S. cerevisiae* was higher than that of *S. uvarum* in all conditions (Figure 3A). Taking the synthetic medium MS200 as an example, *S. cerevisiae* produced 91.6 g/L ethanol (11.6% volume) while *S. uvarum* produced 86.6 g/L (11%). The ethanol yield, arising because of conversion of sugars to ethanol, varied depending on nitrogen source in a similar pattern for both yeasts. Higher ethanol yields when growing on asparagine, aspartate and valine and lower yields on leucine and isoleucine were evident.

S. uvarum was also differentiated from *S. cerevisiae* when central carbon metabolites were considered (Figure 3B). The most notable differences were increased production of glycerol and succinate, and lower formation of acetate and α-ketoglutarate (α-KG) in *S. uvarum*. In addition, an important effect of the nitrogen source on the formation of these metabolites was observed, except for glycerol in which the variation to the mean calculated with data from the other nitrogen conditions was lower than 15% in both species and thus nitrogen effects were subtle. We found that the use of glutamate resulted in a 10-fold increase in α-KG production by *S. uvarum* relative to the mean. The increase factor was 1.67 times for glutamine and aspartate. α-KG formation by *S. cerevisiae* was also largely induced by glutamate and, in a lesser extent, by glutamine and aspartate. Furthermore, the production of succinate was widely affected by the nitrogen source, with

an 8.6 variation factor between the lowest and the highest levels of production by *S. uvarum*. Three groups of nitrogen source were distinguished, showing low (1 g/L in isoleucine), intermediate (~2 g/L in valine, methionine and asparagine) and high succinate production (from 4 to 7.5 g/L in the other nitrogen compounds). Unexpectedly, the response of succinate production to the nitrogen source by *S. cerevisiae* was very different, with a variation factor among all conditions of only 3.7. Moreover, the production of succinate by *S. cerevisiae* was low during methionine and isoleucine fermentation, intermediate when valine, leucine and ammonium were used and high on the rest nitrogen sources, reflecting a different clustering of nitrogen sources in their ability to promote succinate formation than that of *S. uvarum*. Our results showed that, in general, *S. uvarum* produced lower amounts of acetate but with a higher variability depending on nitrogen conditions (from 0.10 to 0.35 g/L), compared to *S. cerevisiae* (from 0.30 to 0.48 g/L). Similar to the formation of succinate, the amino acids triggering the highest acetate production differed between the two species: glutamine and asparagine for *S. uvarum,* and methionine and aspartate for *S. cerevisiae*. Acetate production on methionine was very interesting with opposite effects seen: acetate levels were lowest (of all the single nitrogen sources) in *S. uvarum* but highest in *S. cerevisiae*. Finally, it is noteworthy that the production of central carbon metabolites by *S. uvarum* using a mixture of nitrogen compounds was in the range of variation found using a unique nitrogen source, except for glycerol (overproduced in natural grape juice) and succinate (overproduced in natural and synthetic grape juice). During fermentation on Chardonnay must, acetate was substantially overproduced by both strains.

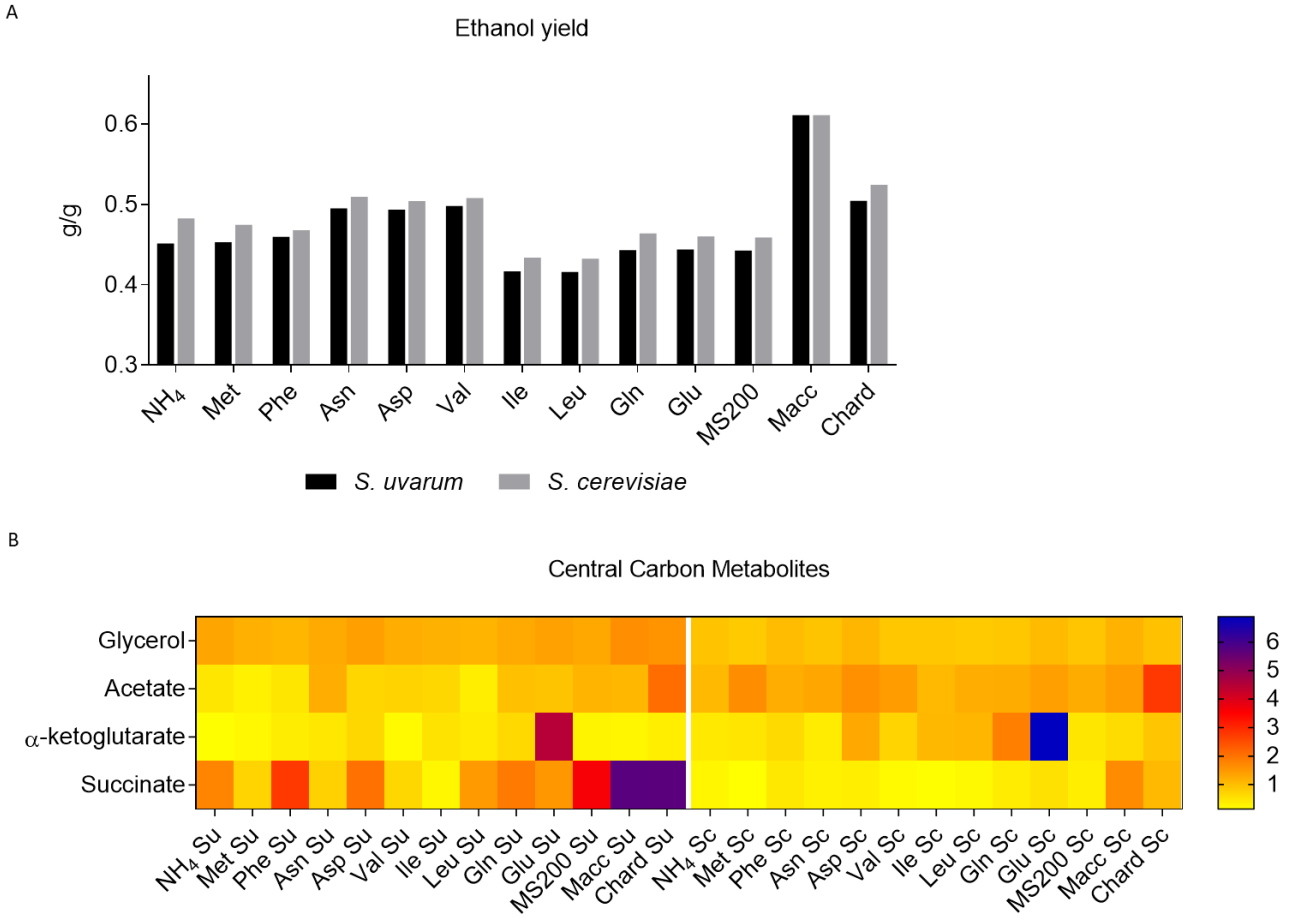

Figure 3. Production of central carbon metabolites by *S. uvarum* and *S. cerevisiae* when growing on different nitrogen sources. Fermentations were conducted with *S. uvarum* (Su) and *S. cerevisiae* (Sc) in 13 different nitrogen conditions: ammonium (NH_4), methionine (Met), phenylalanine (Phe), asparagine (Asn), aspartate (Asp), valine (Val), isoleucine (Ile), leucine (Leu), glutamine (Gln), glutamate (Glu), MS200, Maccabeu (Macc) and Chardonnay (Chard). (A) Ethanol yield of *S. uvarum* and *S. cerevisiae* calculated from biological triplicates, expressed as grams of ethanol produced per grams of sugar consumed. (B) Heatmap of the final production of central carbon metabolites (g/L) normalized with the mean of each compound among all the nitrogen conditions and strains.

Influence of the nitrogen source on the volatile compounds production

To evaluate the influence of the nitrogen source on the aroma profile of *S. uvarum* MTF3098 and *S. cerevisiae* EC1118, the concentrations of volatile compounds at the end of the wine fermentations were compared (Figure 4). A principal component analysis (PCA) revealed that both the species and the nitrogen source, affected their production during fermentation (Supplementary Material 1).

Higher alcohols and their esters derivatives

Higher alcohols and their acetate esters derivatives directly related to the reductive branch of the Ehrlich pathway were differently produced by the two yeast species, and the particular influence of some nitrogen precursors on the production of some volatile compounds was evident. Focusing first on the species effect, comparing the average production for all the amino acids apart from the Ehrlich amino acid precursor, we found overproduction of phenylethanol (4-fold), phenylethyl acetate (17-fold) and 2-methylpropanol (isobutanol) (1.4-fold), and underproduction of methionol (0.7-fold) and propanol (0.5-fold) in *S. uvarum* compared to *S. cerevisiae*. Other volatile compounds in this category like 2-methylpropyl acetate (isobutyl acetate), 3-methylbutanol (isoamyl alcohol) and 3-methylbutyl acetate (isoamyl acetate) were produced in similar concentrations by both strains.

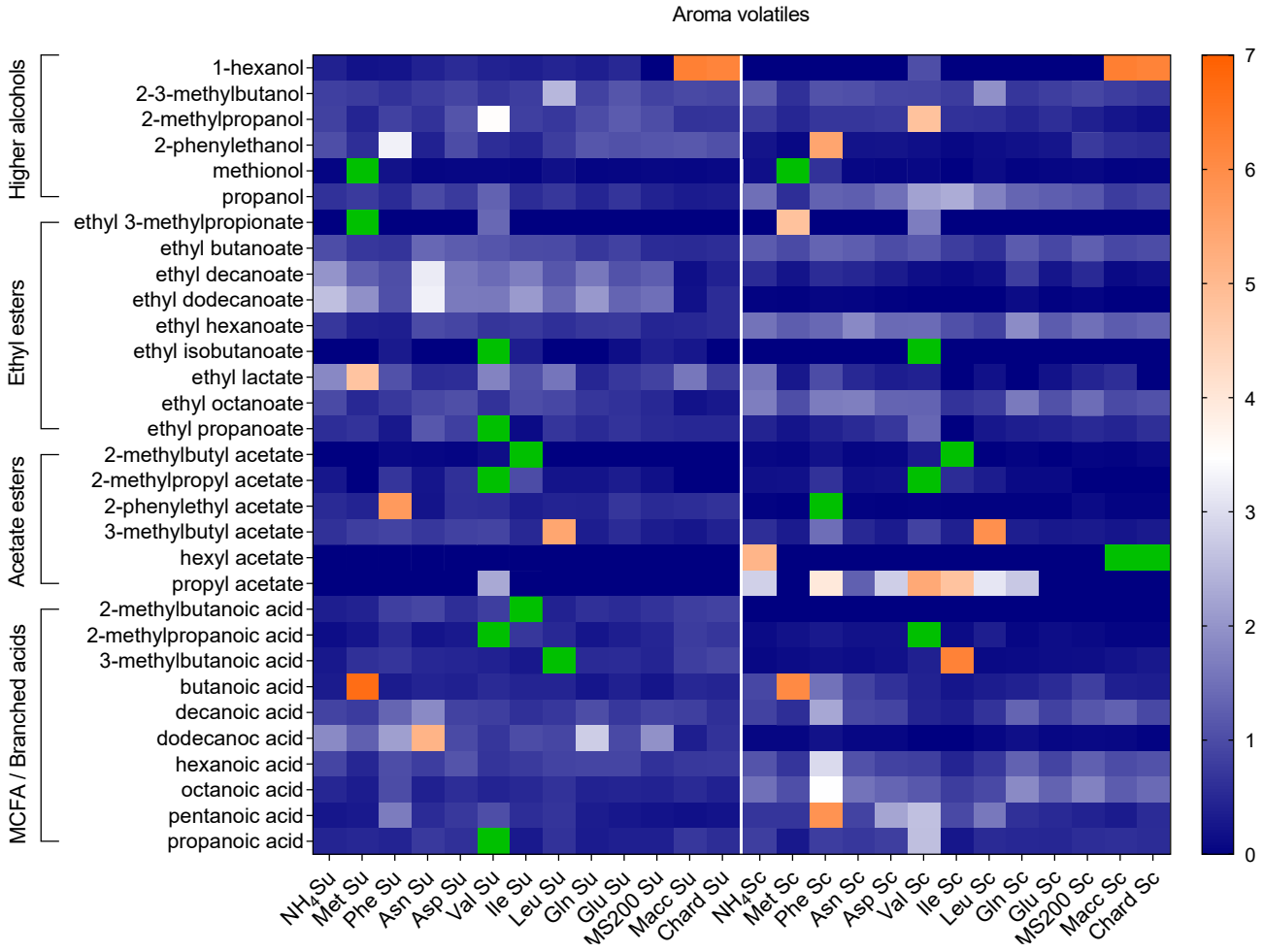

Figure 4. Heatmap summarizing the final production of volatile compounds by *S. uvarum* growing on different nitrogen sources. *S. uvarum* (Su) and *S. cerevisiae* (Sc) fermentations were achieved using 13 different nitrogen conditions providing 200 mgN/L of ammonium (NH_4), methionine (Met), phenylalanine (Phe), asparagine (Asn), aspartate (Asp), valine (Val), isoleucine (Ile), leucine (Leu), glutamine (Gln), glutamate (Glu) as a sole, and MS200, Maccabeu (Macc) and Chardonnay (Chard). Final concentration expressed in mg/L normalized with the mean of each volatile compound produced by both strains in all the nitrogen conditions tested. Green colour represents values higher than 7.

As anticipated, the production of higher alcohols and their acetate ester derivatives was considerably promoted when their specific precursor amino acid was provided as a sole nitrogen source. In *S. uvarum*, increase factors ranging from 3-fold (isoamyl alcohol in presence of leucine compared to the mean of all the other conditions) to 90-fold (methionol in presence of methionine) were found for higher alcohols, and comprised between 7.5-fold (isoamyl acetate in leucine) to 17.7-fold (isobutyl acetate in valine) for acetate esters. In addition, however, some less expected variations in the production of higher alcohols and acetates by *S. uvarum* were triggered by changes of the nitrogen source, like the 2-fold increase of methionol production (compared to the mean value) resulting from the use of phenylalanine or leucine as sole nitrogen source. Another surprising observation was the higher production of phenylethanol and 2-methylpropanol in the presence of glutamate, glutamine, ammonium and aspartate but lower production with the Ehrlich amino acids (apart from phenylalanine and valine, for phenylethanol and 2-methylpropanol, respectively).

Higher acids from branched chain amino acids

Overall, branched-chain carboxylic acids and their ethyl esters derivatives were overproduced by *S. uvarum* compared to *S. cerevisiae*. These compounds derive from the oxidation of the aldehyde intermediates of the Ehrlich pathway, and as seen for the higher alcohols, their formation was promoted when their respective precursor amino acid was provided for growth. Indeed, the production of 2-methylbutanoic, 3-methylbutanoic and 2-methylpropanoic acid was around 20-times increased compared to the mean and their ethyl esters formation was only observed when isoleucine, leucine and valine were used as sole nitrogen sources, respectively. Otherwise, basal production of 2- and 3-methylbutanoic acids (1.7 – 2 mg/L) by *S. uvarum* was found, without a substantial effect of the nitrogen source (less than 40% variation to the mean). Notably, however, 2-methylpropanoic acid was the only branched acid produced by *S. uvarum* that was modulated

by the nitrogen sources other than its direct Ehrlich precursor amino acid (valine). Its formation was increased compared to the mean in the presence of isoleucine, leucine and phenylalanine and conversely decreased on ammonium, glutamine, asparagine, and methionine.

Medium chain fatty acids (MCFA) and MCFA ethyl esters

MCFA from C4 to C12 are produced through the elongation of the carbon chain by the addition of C2 units from acetyl-CoA. For this analysis, the overall MCFA production is reported in µM of acetyl-CoA equivalents, which reflects the pathway activity. In most nitrogen sources, *S. uvarum* produced less MCFA than *S. cerevisiae* (Figure 5). First, the total MCFA formation by *S. uvarum* was affected by the nitrogen source, with low production on valine, isoleucine, leucine, glutamine, glutamate, aspartate, and ammonium (range from 300 to 470 µM), moderate production on phenylalanine (562 µM) and finally, high MCFA production on asparagine and methionine (724 and 723 µM, respectively). The MCFA production profile in *S. uvarum* was also influenced by the nitrogen source. During fermentations with valine, isoleucine, leucine, phenylalanine, glutamate, aspartate, and ammonium the MCFA profile was characterised by a low amount of butanoic acid compared to the other MCFAs. The profile of MCFAs formation obtained with *S. cerevisiae* under the same conditions was quite different, with a major contribution of octanoic acid. The use of methionine as sole nitrogen source triggered the overproduction of butanoic acid by both strains. Finally, in asparagine and glutamine conditions, decanoic and dodecanoic acids were overproduced specifically by *S. uvarum*.

Formation of MCFA ethyl esters was slightly altered by the nitrogen source in a species-dependent way. In *S. uvarum,* the production of ethyl decanoate and ethyl dodecanoate accounted for 84% and 91% of the total production. In *S. cerevisiae*, however, ethyl hexanoate, ethyl octanoate and ethyl decanoate represented between 80% and 88.4% of the total MCFA ethyl esters, with limited production of ethyl butanoate and ethyl dodecanoate. The variation in the total MCFA ethyl esters was low in *S. uvarum,* generally

comprising between 8 and 11.9 mg/L, with slightly decreased production on phenylalanine (6.5 mg/L) and higher production on ammonium (14.2 mg/L) and asparagine (18.7 mg/L). Likewise, the nitrogen source had a weak impact on the formation of MCFA ethyl esters in *S. cerevisiae*, showing low concentrations in Ehrlich pathway amino acids (methionine, isoleucine, and leucine) and higher concentrations in nitrogen sources assimilated through the central nitrogen metabolism (ammonium, glutamine, and asparagine).

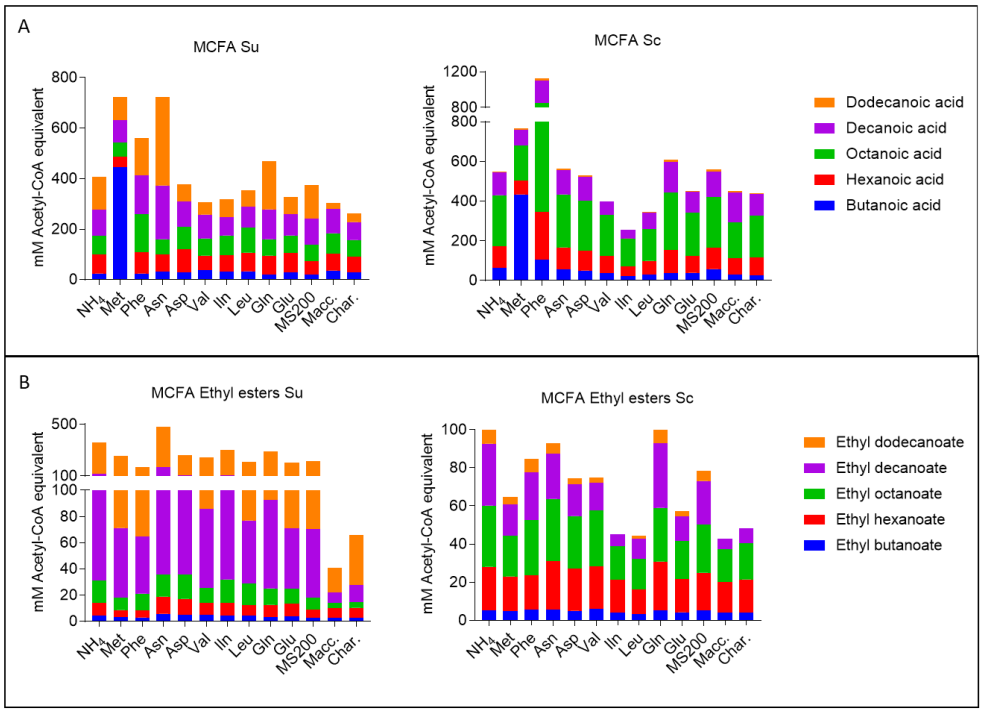

Figure 5. Final production of MCFA and MCFA ethyl esters by *S. uvarum* and *S. cerevisiae* depending on the nitrogen source in the growth medium. Fermentations were conducted using 13 different nitrogen conditions: ammonium (NH_4), methionine (Met), phenylalanine (Phe), asparagine (Asn), aspartate (Asp), valine (Val), isoleucine (Ile), leucine (Leu), glutamine (Gln), glutamate (Glu), MS200, Maccabeu (Macc) and Chardonnay (Chard). Concentration expressed in µM acetyl-CoA equivalent considering the number of acetyl-CoA units required for the synthesis of each compound (2,3,4,5,6 C-units for butanoic, hexanoic, octanoic, decanoic and dodecanoic acids, respectively). (A) Medium chain fatty acids (MCFA). (B) MCFA Ethyl esters.

DISCUSSION

In recent years, the distinctive phenotypic traits of the non-*S. cerevisiae* yeasts, has led to increased interest in considering these as promising

alternative to meet the current challenges of the winemaking sector. New, more sustainable and environmentally-friendly production strategies have to be developed to cope with global warming and indeed to respond to changes in consumer requirements. More widespread and efficient use of these species is restricted, however, because of insufficient knowledge of their metabolic behaviour and no clear understanding of the similarities and differences to *S. cerevisiae*. In this context, we investigated the preferences for nitrogen sources and the effects of these nutrients on the fermentative performances and on the orientation of metabolism in *S. uvarum*.

As a component of proteins and nucleic acids, nitrogen is an essential nutrient for any microorganism and the efficiency of its assimilation strongly shapes their growth capacity and their activity. In *S. cerevisiae*, nitrogen sources related to the central nitrogen metabolic core support growth more efficiently than amino acids of the Ehrlich pathway (Ljungdahl and Daignan-Fornier, 2012; Magasanik and Kaiser, 2002). In this study, a similar classification was demonstrated for *S. uvarum*, showing high specific growth rates on ammonium, glutamate, glutamine and aspartate but lower rates on leucine, valine, phenylalanine and isoleucine. However, important differences were found between the two species in their ability to grow using methionine and asparagine as sole nitrogen source. In *S. cerevisiae*, methionine assimilation is achieved through the Ehrlich pathway and the poor growth compared to other nitrogen sources has been explained by either a low efficiency of the enzymes involved in this metabolic route to retrieve nitrogen for the *de novo* synthesis of proteinogenic amino acids (Gutiérrez et al., 2013), or by an imbalance between enzyme activities resulting in intracellular accumulation of the toxic intermediate 3-methylthiopropionaldehyde (Che et al., 2020; Deed et al., 2019). The relatively better growth of *S. uvarum* on methionine suggests higher efficiency of the transaminases Aro8p and Aro9p or more fine-tuned regulation of the enzymes in this species. Regarding asparagine, the better growth of *S. cerevisiae* could possibly be attributed to the presence of *ASP3*

in some strains, but that cannot be the full explanation since, like *S. uvarum, S. cerevisiae* EC1118 lacks this gene (League et al., 2012). It is also noteworthy that outliers to these species-level findings can be observed at the strain level. In addition to growth, fermentation performance was assessed as the capacity to completely deplete sugar, a trait of interest to the wine industry. Interestingly, the source of nitrogen inversely influenced *S. uvarum* growth and fermentation performance. The amino acids assimilated through the central nitrogen core efficiently support growth but left residual sugar at the end of wine fermentations, whereas Ehrlich amino acids fully used all sugars but only supported slower growth.

There were substantial differences in the formation of central carbon metabolites between the species. The higher production of succinate and glycerol and low production of ethanol in *S. uvarum* has been reported before (Minebois et al., 2020a; Sipiczki, 2008) and is suggested to be a strategy of cryotolerant yeasts to resist low temperatures (Gamero et al., 2013; Lopez-Malo et al., 2013). The high glycerol production, for instance, is explained by the ability of cryotolerant strains to direct carbon flux towards glycerol, whereas *S. cerevisiae* orients the carbon flux towards ethanol production (Arroyo-López et al., 2010). These data also reflect different partitioning of carbon fluxes in the central carbon metabolic network and likely, different strategies for adapting to stresses and constraints of fermentation. *S. uvarum* synthetises glycerol and succinate to ensure energy production and the maintenance of the redox balance, which are essential for growth, while *S. cerevisiae* achieves this through ethanol production. In *S. uvarum*, the highest production of glycerol and succinate was found when Gln, Glu, Asn, Asp and NH_4 were used as sole nitrogen source. Compared to Ehrlich amino acids, these compounds from the nitrogen metabolic core promote more efficient growth, resulting in increased anabolic requirements and *de novo* synthesis of building blocks (in particular proteinogenic amino acids). Consequently, a variable demand in energy and

redox balance management may explain nitrogen source-dependent differences in the formation of central carbon metabolites.

Focusing on the pattern of acetate production, substantial variations were found between the species, most notably lower secretion in *S. uvarum* across all conditions. It has been recently reported that the production of acetate, taking place during the first part of fermentation, is followed by progressive consumption of this compound in *S. uvarum*, but not in *S. cerevisiae* (Minebois et al., 2020a). Furthermore, higher levels of intracellular acetyl-CoA were observed in *S. uvarum* compared to *S. cerevisiae* during wine fermentation (Henriques et al., 2021; Minebois et al., 2020b). There was also a clear impact of the nitrogen source on the formation of acetate in *S. uvarum*, with less secreted acetate when Ehrlich amino acids were used as sole nitrogen source. Under these conditions, growth/fermentation is slower, but the actual fermentation performance as determined by complete utilisation of the available sugars is good. An indication emerging from these studies of the presence of *S. uvarum*-specific management of the acetate metabolic node was reinforced by our findings on the production of acetate-related metabolites.

Compared to *S. cerevisiae*, as well as lower levels of acetate production in *S. uvarum,* there was also a lower amount of excreted total MCFA (as acetyl-CoA equivalent) and an enrichment in C10- and C12-MCFA and their ethyl esters at the expense of compounds with a shorter carbon chain (C4, C6, C8). Taken together, these observations suggest that, in *S. uvarum*, intracellular acetate is mainly directed to the production of MCFAs via acetyl-CoA (Krivoruchko et al., 2015). The reduced levels of secreted shorter MCFAs could indicate their incorporation in membranes to improve plasticity and stress tolerance, rather than excretion or conversion to ethyl esters as is seen in *S. cerevisiae*. This specific trait could be related to the cryophilic phenotype of this species, as an adaptation mechanism to low temperature. Supporting this hypothesis, it was reported that the cryophilic yeast *S. kudriavzevii* has a different lipid composition of the membrane from *S.*

cerevisiae, with higher percentage of MCFAs and shorter fatty acids chain (Tronchoni et al., 2012), which could contribute to membrane fluidity and stress tolerance (Mannazzu et al., 2008; Yang et al., 2019). The higher production of C10 and C12 MCFA ethyl esters by *S. uvarum* was observed regardless the nitrogen source. As fatty acids provide the precursors for these MCFA ethyl esters, this may be related to lipid metabolism, for example via differences in the regulatory enzymes Mgl2 and Yju3 (Marullo et al., 2021). Alternatively, differential expression, activity or substrate specificity of the ethanol acyltransferases Eeb1 and Eht1 (Saerens et al., 2006) may provide the explanation.

S. uvarum produced higher amounts of 2-methylpropanol, phenylethanol and phenylethyl acetate than *S. cerevisiae* regardless of the nitrogen source. There are several possible explanations for this. *S. uvarum* is reported to have a higher flux through the pentose phosphate pathway, which would potentially result in more availability of the shikimate precursor erythrose-4-phosphate (Minebois et al., 2020a, 2020b). Alternatively, it is possible that decarboxylation of phenylpyruvate is preferentially catalysed by Aro10 rather than competing decarboxylases (Pdc1, Pdc5 and Pdc6) (Deed et al., 2019; Li et al., 2017). The recent identification of a new *ARO80* allele, coding for a more efficient transcriptional activator of *ARO9* and *ARO10*, in *S. uvarum* and *S. kudriavzevii,* could support this hypothesis (Tapia et al., 2022). It is also worth noting that Ehrlich volatiles can be synthesised from amino acids catabolism or from CCM as α-ketoacids are a node in both processes. While the highest levels of Ehrlich volatiles are seen when growing on the cognate amino acid (e.g., 8 mM isoamyl alcohol in presence of 14 mM leucine), there is also clear evidence of synthesis via CCM. For example, when growing on a mixture of amino acids the concentration of isoamyl alcohol (2.8 mM) exceeds what could have been provided by the available leucine (0.17mM) by more than a factor of 10. Consequently, it can be concluded that, as with *S. cerevisiae* (Crépin et al., 2017), CCM plays a major role in supplying precursors for synthesis of volatile compounds during

fermentation on natural or synthetic grape must. Furthermore, we observed an overall increased production of the fusel/branched acids 3-methylbutanoic acid and 2-methylbutanoic acid by *S. uvarum* compared to *S. cerevisiae*. The promotion of fusel acids formation in *S. uvarum* may be explained by a more efficient activity of aldehyde dehydrogenase (ALD) enzymes (Boer et al., 2007; Vuralhan et al., 2005), or by differences in the redox balance (NAD+/NADH+ pools) between the two species.

In conclusion, through the examination of associations between the nature of nitrogen source and the fermentation performance of *S. uvarum*, we demonstrate both similarities and differences to *S. cerevisiae*. The distinctive profile of central carbon metabolites and volatile compounds of *S. uvarum* compared to *S. cerevisiae,* highlighted its unique distribution of carbon fluxes in the metabolic network to fulfil anabolic requirements and maintenance of redox balance. These peculiarities are exacerbated depending on the nitrogen source, in relation to the efficiency of amino acids and ammonium to support growth and fermentation. This is essential knowledge that should be considered in order to exploit the phenotypic potential offered by *S. uvarum* in winemaking and more widely in the food and beverage industry.

Supplementary information The online version contains supplementary data available at DOI:10.5281/zenodo.6627770.

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Conflict of Interest The authors have no relevant financial or non-financial interest to disclose.

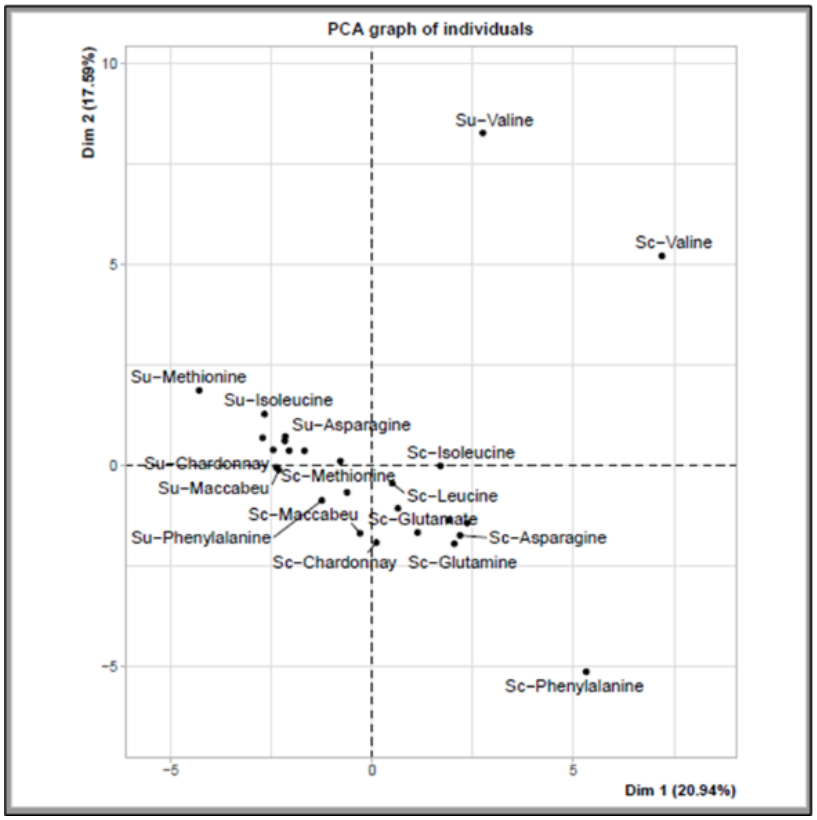
SUPPLEMENTARY MATERIAL

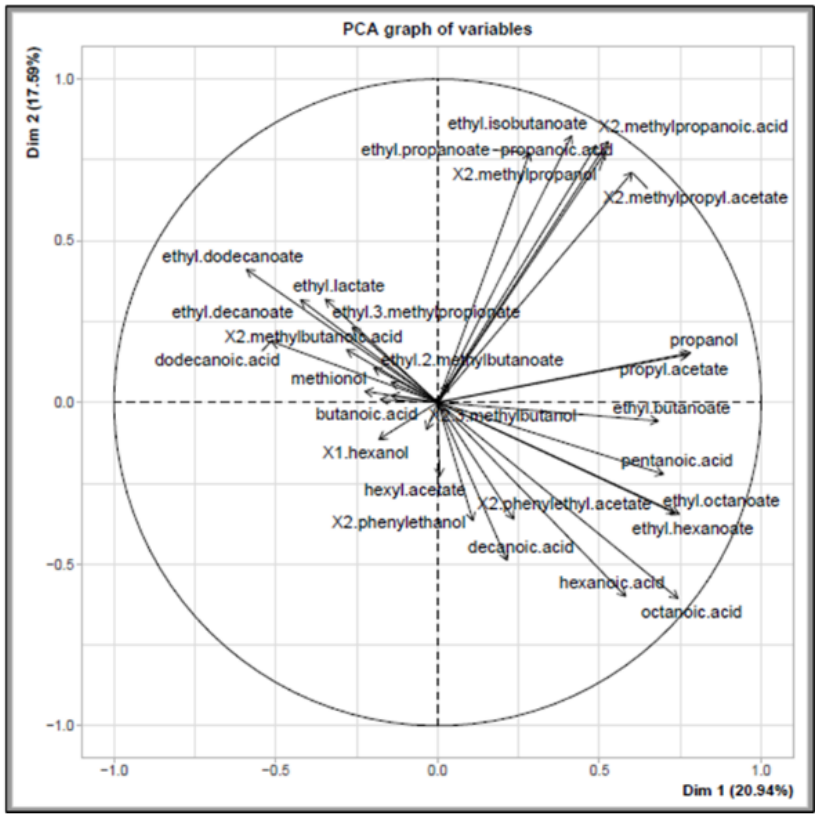
Supplementary Table 1. Growth parameters calculated from wine fermentations. Data used for construction of Figure 2C.

Strain	Nitrogen Condition	Growth parameters (Relative to mean)			
		Rmax	TimeAtRmax	CO2atRmax	LagTime
S. uvarum	Ammonium	0.97	0.77	0.80	0.75
	Methionine	0.94	0.97	0.89	1.00
	Phenylalanine	0.90	1.00	0.87	1.04
	Asparagine	0.71	1.60	1.20	1.58
	Aspartate	0.97	0.94	0.90	0.96
	Valine	0.93	1.15	1.08	1.17
	Isoleucine	0.81	1.73	1.20	1.89
	Leucine	1.04	0.99	0.93	1.07
	Glutamine	0.82	0.71	0.66	0.67
	Glutamate	0.90	0.93	1.14	0.74
	MS200	0.94	0.64	0.78	0.53
	Maccabeu	0.75	0.71	0.70	0.60
	Chardonnay	0.89	0.69	0.87	0.53
S. cerevisiae	Ammonium	2.46	0.78	0.84	0.84
	Methionine	1.26	1.33	1.35	1.48
	Phenylalanine	1.11	1.11	0.98	1.19
	Asparagine	1.20	0.77	0.81	0.83
	Aspartate	1.18	0.79	0.84	0.84
	Valine	1.34	1.26	1.58	1.34
	Isoleucine	1.13	2.45	2.23	2.73
	Leucine	1.30	1.15	1.35	1.22
	Glutamine	1.20	0.76	0.99	0.79
	Glutamate	1.01	0.86	0.76	0.94
	MS200	1.13	0.63	0.97	0.48
	Maccabeu	0.91	0.74	0.90	0.56
	Chardonnay	1.02	0.63	0.89	0.48

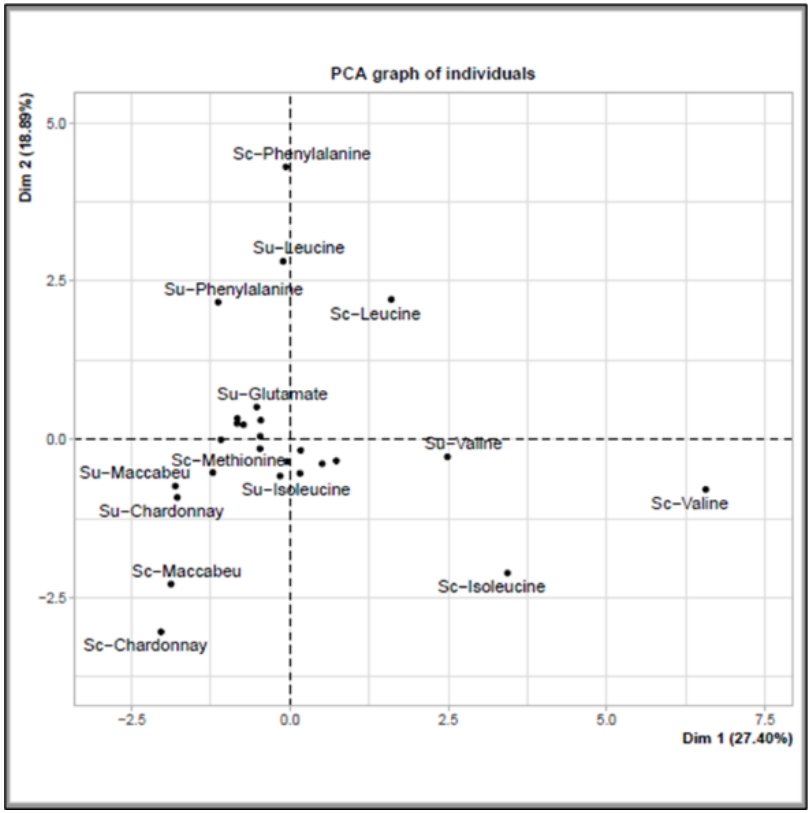
Supplementary Material 1.

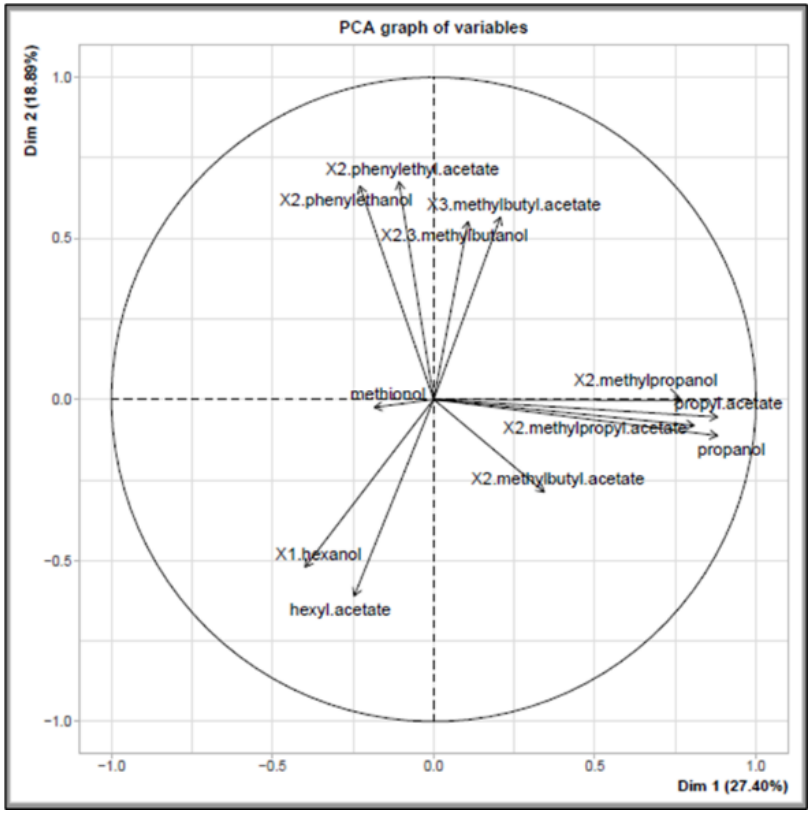
All volatile compounds



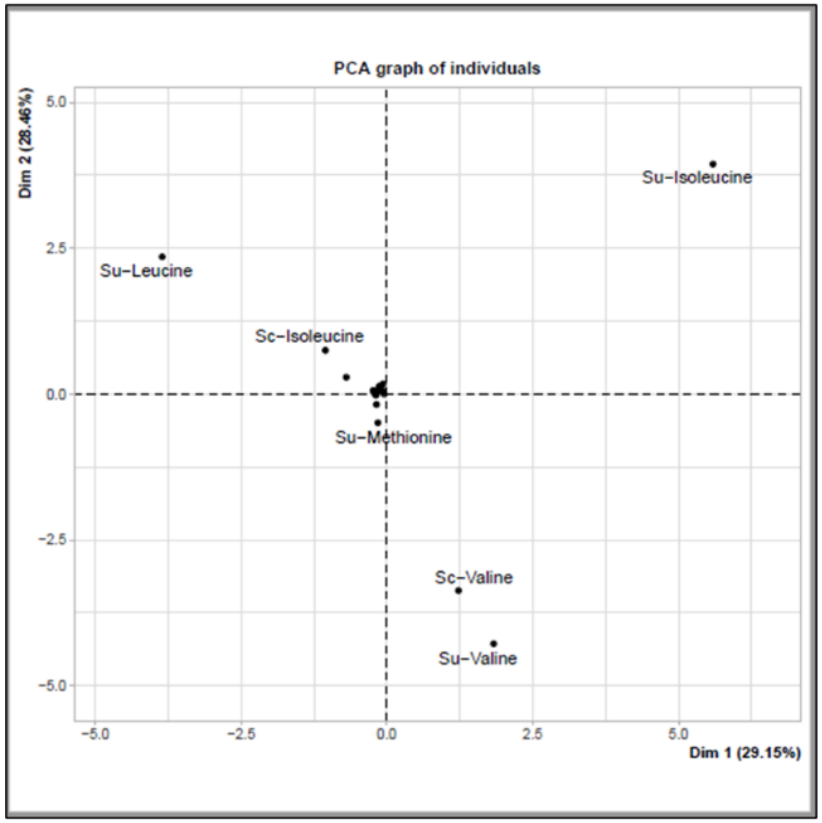


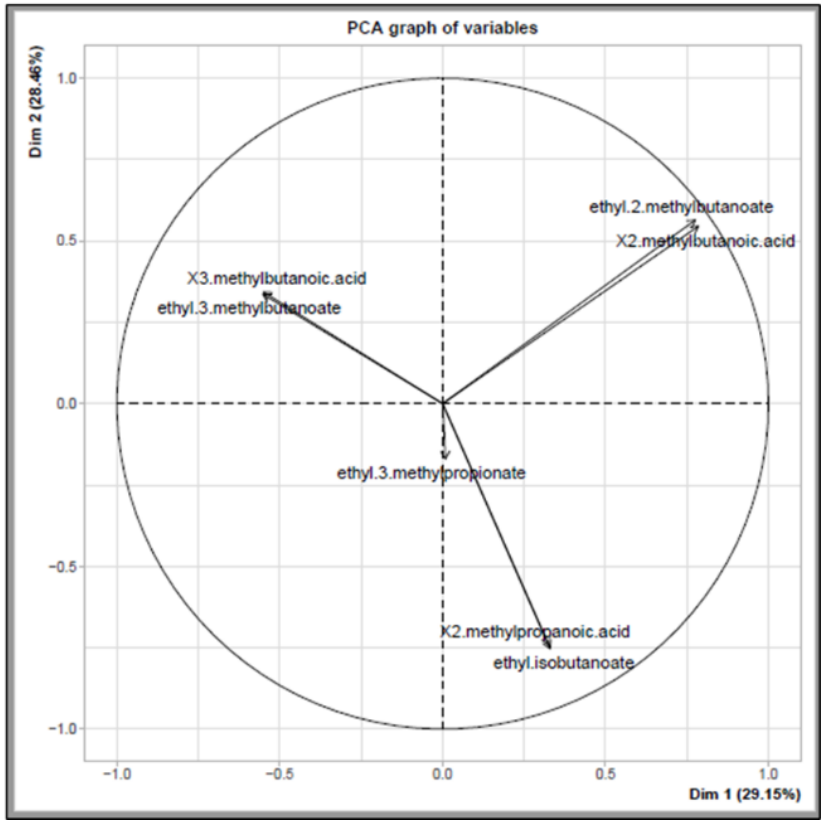
Alcohols and acetates



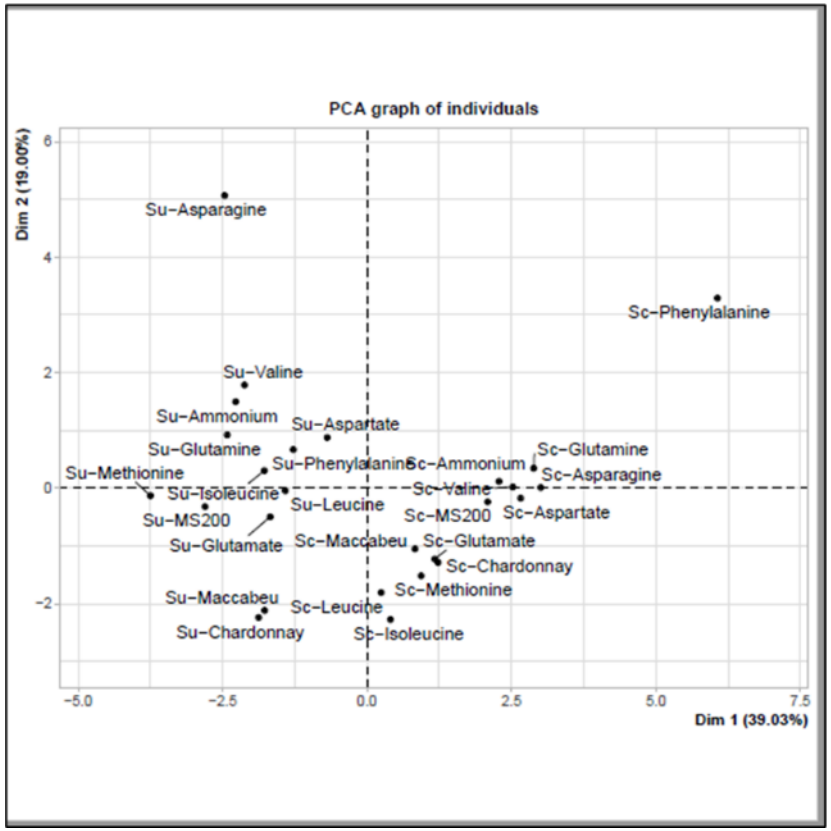


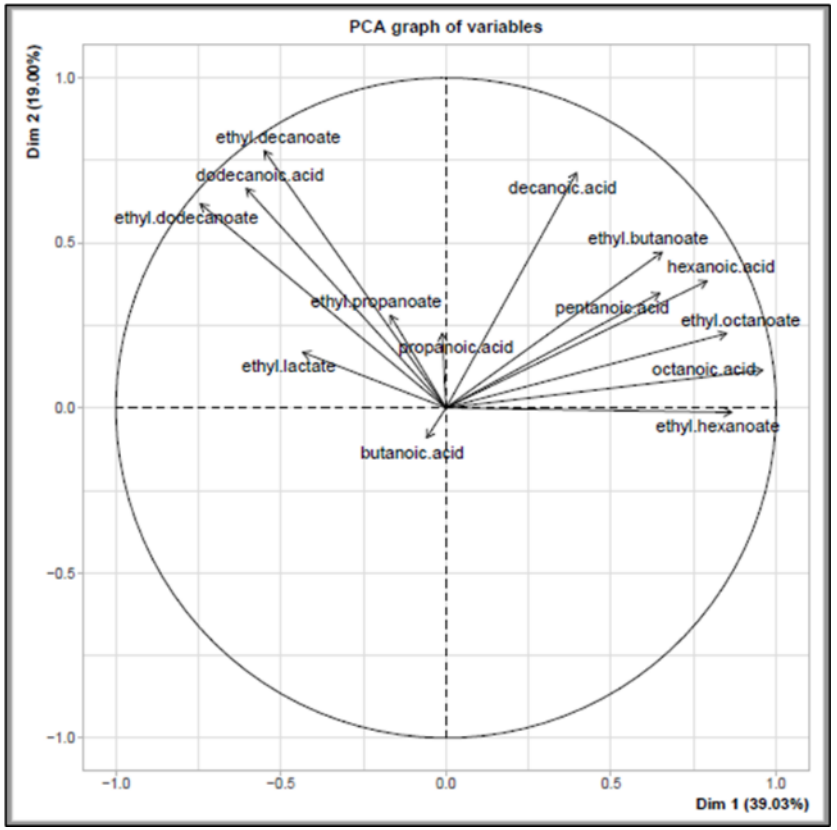
Branched acids





MCFA and esters





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Chapter 2

Transcriptional landscape of *Saccharomyces uvarum* when growing using preferred and non-preferred nitrogen sources

Angela Coral-Medina[1,2], Darren A. Fenton[1,3], Carole Camarasa[2], John P. Morrissey[1,4]

[1]School of Microbiology, University College Cork, Ireland; [2]SPO, Univ Montpellier, INRAE, Institut Agro, Montpellier, France; [3]School of Biochemistry and Cell Biology, University College Cork, Ireland [4]Environmental Research Institute and SUSFERM Fermentation Science Centre, University College Cork, Ireland

Corresponding author: j.morrissey@ucc.ie

ABSTRACT

Saccharomyces uvarum is used for the production of ciders and some white wines. It is a cryotolerant yeast, capable of growing at lower temperatures than *Saccharomyces cerevisiae*, which is particularly beneficial for apple juice fermentations. It is also reported to produce high levels of aromatic volatiles, making it very attractive for modern beverages. Many of these volatiles have their origins in nitrogen metabolism, which is also important for growth as nitrogen is generally the limiting nutrient in apple or grape musts. Because of its dual importance, nitrogen metabolism has been extensively studied in *S. cerevisiae* where there is a vast amount of knowledge on metabolic pathways and regulator processes. At a macro level, similar pathways and processes can be predicted to operate in *S. uvarum* given a broadly orthologous gene content. Nonetheless, as it is known that the yeasts neither grow identically, nor have identical profiles of volatile aromatics, it follows that there must be differences in nitrogen metabolism. There are little data on this topic so in this study, we decided to establish a base-line to understand this topic by studying the transcriptome of *S. uvarum* when growing on different nitrogen sources, namely ammonium sulphate and amino acids that were either preferred or non-preferred nitrogen sources. Through this analysis, we established that regulator systems such as the SPS sensor and nitrogen catabolite repression (NCR) play a major role when cells are growing on non-preferred nitrogen sources. This is analogous to *S. cerevisiae,* but we also found some start differences, notably regarding the response of the cells to asparagine, which is a preferred nitrogen source in *S. cerevisiae* but not in *S. uvarum.* Interestingly, while much of the responses to amino acids that were seen related to specific metabolic pathways, there was also a discernible signal for non-preferred nitrogen sources. We complemented the transcriptome analysis with metabolome data and found that central carbon metabolism was also a factor that influenced aroma outcomes. This study increases understanding of the importance of the nitrogen source in the aroma

production of *Saccharomyces* yeasts and broads the knowledge on *S. uvarum* nitrogen metabolism in relation to its attractive aroma profile.

INTRODUCTION

In this study, we focused on *Saccharomyces uvarum* which is an important yeast in the *Saccharomyces* genus (Rainieri et al., 1999), commonly isolated from cold environments and used for the production of white wine, cider and other fermented beverages (Almeida et al., 2014; González Flores et al., 2017; Pérez-Torrado et al., 2015). *S. uvarum* strains have been isolated from different geographical origins across the globe; they are widespread in natural environments and other environments associated with human-driven fermentations (Albertin et al., 2018; Masneuf-Pomarede et al., 2016). According to Almeida et al., (2014) *S. uvarum* is native to the Southern Hemisphere and its global phylogeography is explained by its association with the *Nothofagus* tree genus. Moreover, *S. uvarum* hybridize with other *Saccharomyces* species such as *S. eubayanus, S. pastorianus*, and *S. cerevisiae*, and such hybrids are important in the brewing industry especially due to the capacity to ferment at low temperatures conferred by *S. uvarum* (Nguyen et al., 2011). Importantly, *S. uvarum* differs from *S. cerevisiae* even though they are closely genetically related species. Various studies have assessed the fermentation profile of *S. uvarum* in wine conditions compared to *S. cerevisiae* and found enhanced fermentation performance at low temperature, lower ethanol and acetic acid production, and increased production of glycerol, succinate and aroma compounds (Alonso-del-Real et al., 2017; González Flores et al., 2017; Minebois et al., 2020; Pérez-Torrado et al., 2015; Varela et al., 2016). While *S. uvarum* is an alternative species used for wine fermentation, it has the main role in cider fermentations.

Inefficient nitrogen assimilation results in slow and sluggish fermentations (Blateyron and Sablayrolles, 2001) that cause issues in the fermentation industry. There is a large number of published studies describing the

preference of nitrogen sources used by yeast. Nitrogen compounds are classified as preferred or non-preferred depending on different criteria, such as the efficiency to sustain growth, the effect on regulation mechanisms and the order of consumption when provided in mixture with other nitrogen compounds. Although the classification has been empirical and literature differs on its definition, most studies consider preference to be the ability to support growth (Magasanik and Kaiser, 2002). In addition, however, besides its influence on growth, the nitrogen source provided as ammonium and amino acids, influences aroma synthesis during fermentation. Amino acids are precursors of aroma compounds and influence other metabolic processes, such as the expression of regulators of purines and pyrimidines for nucleotides formation, the biosynthetic pathway of other amino acids, and the nitrogen regulation pathways that affect the entire cellular metabolism (Ljungdahl and Daignan-Fornier, 2012).

Among the most important regulation mechanisms is Nitrogen Catabolite Repression (NCR), which prevents the expression of transporters and catabolic enzymes that assimilate non-preferred nitrogen compounds in the presence of preferred nitrogen sources (Hofman-Bang, 1999). NCR involves GATA transcription factors (Gln3p, Gat1p, Dal80p and Gzf3p) that activate or repress gene expression by binding -GATA- sequences in nitrogen-regulated promoters (Cunningham and Cooper, 1991; Stanbrough et al., 1995). Magasanik and Kaiser (2002), proposed that NCR mainly results in the alteration of the nitrogen uptake rate through the permeases expression depending on the intracellular pools of glutamine and glutamate; in that way, the uptake rate of precursors of glutamine and glutamate is regulated. Moreover, there is a contribution from the SPS-sensor system to nitrogen uptake (Zhang et al., 2018). The SPS-sensor controls amino acid transport by upregulating the expression of amino acid permeases (AAPs). This pathway involves the primary sensor (Ssy1, Ptr3 and Ssy5) that recognizes extracellular amino acids and sends a signal to the downstream components, which then interact with the permease's promoter to induce

transcription (Ljungdahl, 2009). The regulation pathways interconnect to respond to environmental cues. The central regulation pathway that overlap with the SPS and NCR systems is the target of rapamycin (TOR) pathway that controls cell growth and metabolism in response to nutrients (Schmelzle and Hall, 2000). TOR indirectly interacts with the SPS signalling and NCR factors, and it is sensitive to nitrogen availability, triggering changes in nitrogen assimilation proteins and altering the magnitude of the transcriptional response. Therefore, TOR pathway acts independent and in parallel to contribute to the expression of the involved factors (Godard et al., 2007; Ljungdahl, 2009; Ljungdahl and Daignan-Fornier, 2012). Finally, another nitrogen regulation mechanism related to yeast growth is the general amino acid control (GAAC) system that controls genes involved in amino acid biosynthesis in nitrogen starvation conditions. The core factor of this biosynthetic pathway is Gcn4p, which is induced in the absence of amino acids to activate transcription of biosynthetic genes by binding the UAS_{GCRE} consensus sequence of more than 1000 target genes (Natarajan et al., 2001). The literature on nitrogen regulation of *S. cerevisiae* determined that the nitrogen requirements depend on the strain, but overall, the regulation mechanisms are rather conserved within the species. It remains to be determined whether this also applies to other *Saccharomyces spp*.

While nitrogen regulation and its relationship with production of aroma compounds is well described in *S. cerevisiae*, relatively little is known about *S. uvarum* metabolism in different nitrogen sources, nor the precise genetics that are responsible for the attractive aroma profile provided by this yeast. The purpose of this investigation was to explore nitrogen metabolism in *S. uvarum* and the effects of the nitrogen source on the production of aroma volatiles at lower temperature since this is relevant for production of wine or cider. For this study, the commercial strain *S. uvarum* MTF3098, which has been previously characterized in many studies, was used as representative of the species (Alonso-del-Real et al., 2017; Gamero et al., 2013; Minebois, 2017). The nitrogen sources selected were ammonium

(NH_4), asparagine, phenylalanine and methionine based on experiments carried out by Boer et al., (2007) that classified these sources as good (NH_4 and Asn), intermediate (Phe), and poor (Met) in *S. cerevisiae.* Methionine and phenylalanine are catabolised through the Ehrlich pathway, unlike asparagine and ammonium that are directly incorporated into the central nitrogen metabolism. This paper begins by describing the fermentation kinetics in different nitrogen conditions. Then it goes on to reveal the transcriptomic and metabolomic analysis in the same conditions and find the association of expressed genes and the aroma metabolites produced. This study increases understanding of the importance of the nitrogen source in the aroma production of *S. uvarum*, with applications in the food and beverage industries.

MATERIALS AND METHODS

Strains and culture media

All yeast strain used in this study are listed in table 1. For each set of experiments, strains were recovered from -80°C glycerol stocks in YPD broth (2% glucose, 2% peptone, 1% yeast extract) for 24 hours at 28°C. Pre-cultures for fermentation were grown in YPD broth for 16 hours and then transferred to Yeast Nitrogen Base (YNB) media without amino acids and ammonium for 4 hours at 28°C to exhaust the yeast nitrogen reserves. Cells were washed three times with 0.9% NaCl to remove all nitrogen residues and then resuspended in the test media without a nitrogen source. The minimal media (MM) used was adapted from Verduyn media (Verduyn et al., 1992) and contained glucose 20 g/L, yeast assimilable nitrogen (YAN) 1059 mgN/L (the precise concentration of the nitrogen compound varied as described below), KH_2PO_4 3 g/L, $MgSO_4.7H_2O$ 0.5 g/L, vitamin mix, and trace elements.

The nitrogen conditions tested were ammonium sulfate 5 g/L, asparagine 5 g/L, methionine 11.2 g/L and phenylalanine 12.5 g/L, concentrations that

correspond to 1059 mgN/L of YAN to provide equal amounts of nitrogen. When the nitrogen source was supplied as single amino acid, the medium was supplemented with K_2SO_4 6.6 g/L to provide comparable amounts of SO_4 to the ammonium medium.

For characterising the growth of wild-type and mutant *S. uvarum* strains on different nitrogen sources, the strains were grown in minimal media individually, containing ammonium, methionine, phenylalanine, asparagine, aspartate, glutamine, glutamate, valine, isoleucine, leucine and proline as sole nitrogen sources in concentrations correspondent to 1059 mgN/L of YAN.

Construction of mutants

CRISPR-Cas9 technology was used to delete *YJL213W* copies in *S. uvarum* MTF3098 yielding the single and double mutants listed in Table 1. The target sequence identification for gene deletions was performed manually. Primers encoding the target sequences and specific overhangs (Supplementary Table 1) were annealed and cloned into pUDP002-HH-BsaI with Golden Gate Assembly method as previously described (Rajkumar et al., 2019). The BSA-R primer was used in combination with the target forward primer to check for correct plasmid assembly in NEB 5-alpha *E. coli* colonies. Plasmids containing the target sequence were extracted and transformed into *S. uvarum* using the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl, 2007). The gene deletion was done using repair fragments listed in Supplementary Table 1. The deletions were 1096 bp long in *YJL213W-1* and 917 bp long in *YJL213W-2* which disrupted the genes. The mutant strains were checked by PCR with diagnosis primers and DNA sequencing (Eurofins Genomics, Germany).

Growth and fermentations

Batch fermentations were performed in 330 mL fermenters containing 250 mL of media, equipped with fermentation locks to prevent the entry of oxygen while allowing the release of CO_2. The fermentations were

performed in biological triplicates at 20°C with continuous stirring at 230 rpm. The initial population in each fermenter after inoculation was ~0.08 A_{600}. The fermentations were monitored by CO_2 release, weighing the fermenters to track weight loss. Sampling points were determined depending on the growth and consisted of three types of sampling depending on the post-fermentation experiments. For the frequent sampling, 1.5 mL of media were taken every three hours and A_{600} measured to monitor the cell population size. The samples were then centrifuged at 13000 rpm for 5 minutes at 4°C and the supernatant was stored at -20°C for later HPLC analysis. 6 mL samples destined for GC-MS analysis were taken at the end of fermentation and treated in the same way as the aforementioned HPLC samples.

For the transcriptome evaluation of *S. uvarum* in different nitrogen conditions, the samples were taken at the ~0.25 fermentation progress point (25% of consumed sugar) based on the fermentation activity and glucose consumption in each condition. At the sampling time, 25 mL of media were centrifuged at 5000 rpm for 10 minutes at 4°C, the supernatant was discarded, and the cell pellet was snap-frozen in liquid nitrogen. The pellets were stored at -80°C for later RNA extraction.

Analysis of growth phenotype of *S. uvarum* wild type and mutant strains was done in a 96 flat wells microplate incubated in the microplate reader CLARIOStar®^{Plus} (BMG LABTECH, Germany). The absorbance at 600 nm was measured for 60 cycles of 1 hour (24 flashes/cycle) with continual double orbital shaking (400 rpm) between measurements. Growth kinetics data was analysed using R Studio software, version 1.3.1093 (RStudio Team., 2020) with GrowthCurver package (Sprouffske and Wagner, 2016). The graphs and statistical tests for growth and metabolites production were performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California, USA). We report area under the curve (AUC) values as a metric of growth (Su et al., 2020).

RNA extraction and sequencing

Total RNA was extracted following the Hot Phenol RNA Extraction method adapted from (Wilhelm and Martin, 2017). Pellets were reconstituted in lysis buffer (EDTA 0.5 M, NaOAc 3 M, pH 5.5) and SDS 20%. For extraction, hot acid phenol:chloroform (Phenol-Chloroform-Isoamyl alcohol mixture, pH 4.5, ratio 125:24:1) (Sigma-Aldrich, MI, USA) was added to the samples and heated at 65°C. Isopropanol and iced ethanol 80% were used for precipitation and washing steps respectively. Glycoblue 150 µg/mL (Thermo Fisher Scientific Inc.) was used as co-precipitant for pellet visualization. The RNA pellet was reconstituted in Rnase-free water and then quantified with Qubit RNA HS Assay Kit (Thermo Fisher Scientific Inc.). The quality control of RNA samples was done at Beijing Genomics Institute (BGI Tech Solutions, Hongkong, China) using the Agilent 2100 Bioanalyzer with the RNA 6000 nano Kit and Nanodrop Spectrophotometer for quantification. The transcriptome library preparation and RNA Sequencing was done by BGI Tech Solutions using the DNBSEQ™ sequencing technology.

RNA sequencing data analysis and bioinformatics

De novo assembly of *S. uvarum* MTF3098 transcriptome from RNA-seq data was performed using Trinity Software (Grabherr et al., 2013). To obtain a uniform reference transcriptome among samples, all reads were first pooled together for assembly. After *de novo* assembly, the reads of each sample were individually aligned back to the reference transcriptome. The transcriptome analysis was done using DESeq2 R package (Love et al., 2014) that generates Stat scores calculated with the Wald Test and then p-values adjusted for multiple testing using Benjamini-Hochberg (BH) method (Benjamini and Hochberg, 1995). The DESeq2 package generates pairwise comparisons of each amino acid with ammonium as control. For the transcriptome analysis, differences in gene expression were considered statistically significant when the adjusted p-values were <0.01. The fold change cut-off was <-1.5 and >1.5 to identify differentially expressed genes.

The gene expression data was analysed and graphed using R Studio software, version 1.3.1093.

Functional analysis was performed on selected set of genes to identify significant biological process (BP) corresponding to the Gene Ontology terms (GO Terms) annotation with the GeneCodis4.0 program (García-Moreno et al., 2021). Finally, the YJL213W protein sequence of *S. uvarum* was analysed with the SWISS-MODEL server (Waterhouse et al., 2018) to get functional information about the protein and the predicted structure.

Metabolite analytic methods

The measurement of glucose, ethanol, glycerol and organic acids was done with High-Performance Liquid Chromatography (HPLC) on Phenomenex Rezex ROA column (HPLC HP1100 Infinity, Agilent Technologies) as described by Rollero et al., 2015. In addition, to measure volatile compounds at the end of fermentation, a liquid extraction was first done with dichloromethane CH_2Cl_2, then the organic phase was recovered, dried, and concentrated with nitrogen gas before injection. The volatiles measurement was performed by gas chromatography/mass spectrometry (GC-MS) with a Hewlett Packard 6890 gas chromatograph (Agilent Technologies, Santa Clara, California, USA) equipped with a CTC Combi PAL Autosampler AOC-5000 (Shimadzu, Columbia, USA), coupled to a HP 5973 mass spectrometry detector (HP, now Agilent Technologies, Santa Clara, California, USA) as described in detail by Rollero et al., 2015.

Table 1. Strains used for the study. Location of deletions in mutant strains used the co-ordinates from *S. uvarum* CBS7001.

Yeast	Strain	Genotype of mutants	Reference
S. cerevisiae	CEN.PK113-7D	Wild type	Nijkamp et al., 2012
S. uvarum	BMV58/MTF3098	Wild type	Velluto BMV58™ from Lallemand Inc.
S. uvarum	*Δyjl213w-1*	CHR I Δ197,889 – 196,794	This study
S. uvarum	*Δyjl213w-2*	CHR VI Δ521,898 – 520,982	This study
S. uvarum	*Δyjl213w-1* *Δyjl213w-2*	CHR I Δ197,889 – 196,794; CHR VI Δ521,898 – 520,982	This study

RESULTS

S. uvarum differs from *S. cerevisiae* in nitrogen source consumption

Given the importance of nitrogen metabolism for growth and fermentation performance, we first investigated the use of different nitrogen sources by *S. uvarum.* The choice of ammonium sulphate, methionine, phenylalanine and asparagine reflected prior work in *S. cerevisiae* where the nitrogen preference is known. *S. cerevisiae* and *S. uvarum* were cultured for 48 hours with the different nitrogen sources and overall growth was determined using the parameter "area under the curve" that captures growth rate as well as population size (Figure 1). The findings for *S. cerevisiae* reflected prior studies, with ammonium and asparagine being better sources of nitrogen than methionine and phenylalanine. The data for *S. uvarum* were similar with the exception of asparagine, which was not an efficient nitrogen source for this yeast. To explore *S. uvarum* fermentation performance in more detail, a comprehensive analysis was also carried out by measuring production of ethanol and CO_2, consumption of glucose, as well as increase in population size (Figure 2). It was found that ammonium efficiently supports growth, phenylalanine and methionine sustain intermediate growth, while the lowest growth was observed in asparagine. As a consequence of the impact of the nitrogen source on the fermentative activity of *S. uvarum*, the duration of fermentation varies from 36 hours on ammonium to over 48 hours on asparagine. Interestingly, although the rate of fermentation, as monitored by the rate of CO_2 production, was faster on ammonium than methionine or phenylalanine, the final ethanol and biomass yields were largely the same under all three conditions. Values were lower on asparagine as, even after 48 hours, the fermentation was not completed. Taken together, these data show that different nitrogen sources affect the overall fermentation in specific ways, and this led us to further investigations through differential analysis of gene expression during fermentation on these nitrogen sources.

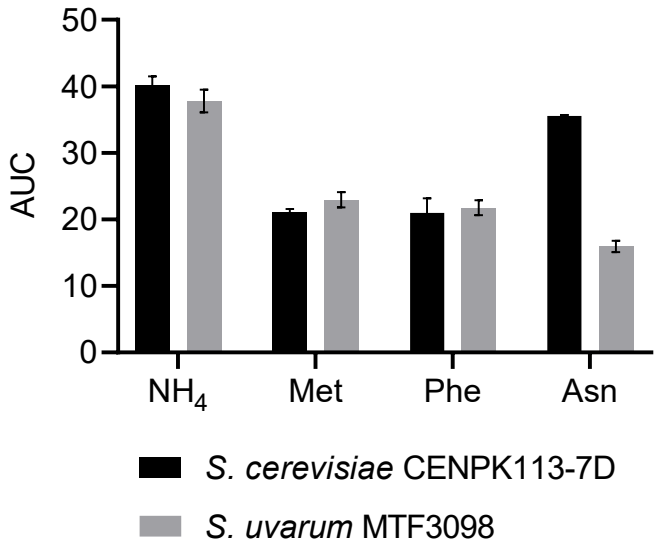


Figure 1. Growth of *S. uvarum* and *S. cerevisiae* in ammonium (NH_4), asparagine (Asn), methionine (Met) and phenylalanine (Phe) as sole nitrogen sources. Fermentations done in minimal medium during 48 hours at 20°C. The area under the curve (AUC) values represents the maximum biomass produced, maximum specific growth rate and the lag phase. The error bars show the standard deviation (SD) from biological triplicates.

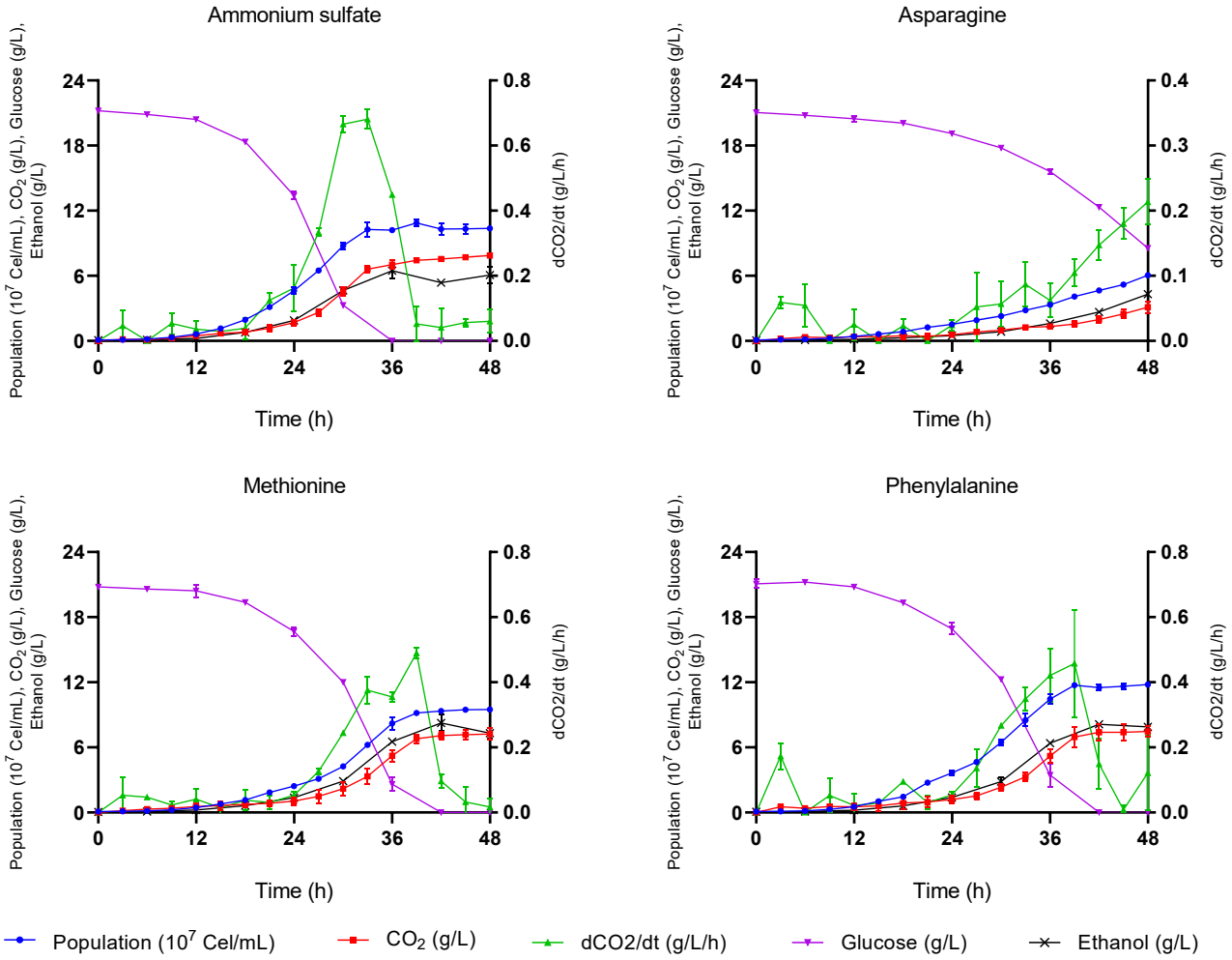


Figure 2. Fermentation profile of *S. uvarum* in ammonium (NH_4), asparagine (Asn), methionine (Met) and phenylalanine (Phe) as sole nitrogen sources. Fermentations done in minimal medium during 48 hours at 20°C. The parameters measured were population (10^7cel/mL), CO_2 production (g/L), CO_2 production rate (g/L/h), glucose consumption (g/L), and ethanol production (g/L). Mean values were calculated from biological duplicates.

Influence of the nitrogen source on the gene expression: Overview of the dataset

The first step to achieve a comparative analysis of gene expression profile, was to identify the conditions in which the cells were in the same physiological state to allow meaningful comparison between the nitrogen conditions. Since the growth dynamics of *S. uvarum* is very different depending on nitrogen source, we compared the gene expression profiles at 25% fermentation progress, when the cell physiology, growth phase and other stresses were the same in all conditions. Exploratory batch fermentations were done in MM with each nitrogen source to obtain the fermentation time required to reach 25% fermentation progress when 5 g/L of the 20 g/L of glucose initially contained in the media had been consumed (Supplementary Figure 1). This information was used in the main fermentations, where samples were taken at 25% fermentation progress, and the transcriptome and metabolome analysed as described in the methods. At the time of analysis, there was not a good reference genome for *S. uvarum* available, so we used the RNA-Seq data to perform *de novo* assembly with Trinity software (see methods). Total sequence reads varied from 49 to 51 million per condition, all of the total clean reads ratio were high (>97.5%), and a total of 5171 genes were detected for each condition.

Pairwise comparisons with ammonium-grown cultures were performed to elucidate the response triggered by amino acids. On the question of what is the transcriptional common response triggered by the tested amino acids relative to ammonium, we analysed the genes with the highest scores and evaluated the enrichment in GO Terms Biological Process (BP) database. The Venn diagrams (Figure 3) show the relationship of expressed sets of genes among methionine, phenylalanine and asparagine, and the bar plots represent GO Terms annotations. Gene lists are available in Supplementary file. As shown in Figure 3, there was a small number of common up-regulated genes (24) and very few common downregulated genes (2) among the conditions, suggesting that the amino acids trigger a unique response.

The 24 genes that overlap in the up-regulated group are involved in transmembrane transport, associated with the SPS-sensor pathway that controls the amino acids uptake by inducing the transcription of amino acid permeases (AAPs). There was also significant up-regulation of genes associated with the metabolic processes of different nitrogen compounds such as urea, ornithine and BCAA.

It was not surprising that, a much larger transcriptomic response (up and down-regulation) was observed in asparagine than methionine or phenylalanine given the previous fermentation data that revealed significant growth differences. Additionally, in methionine there was almost equal number of up and down-regulated genes, whereas phenylalanine had twice as many up-regulated genes as methionine and few down-regulated genes. This indicates that even though fermentation performance was similar, substantial differences exist at gene expression level, underlying changes happening in metabolism.

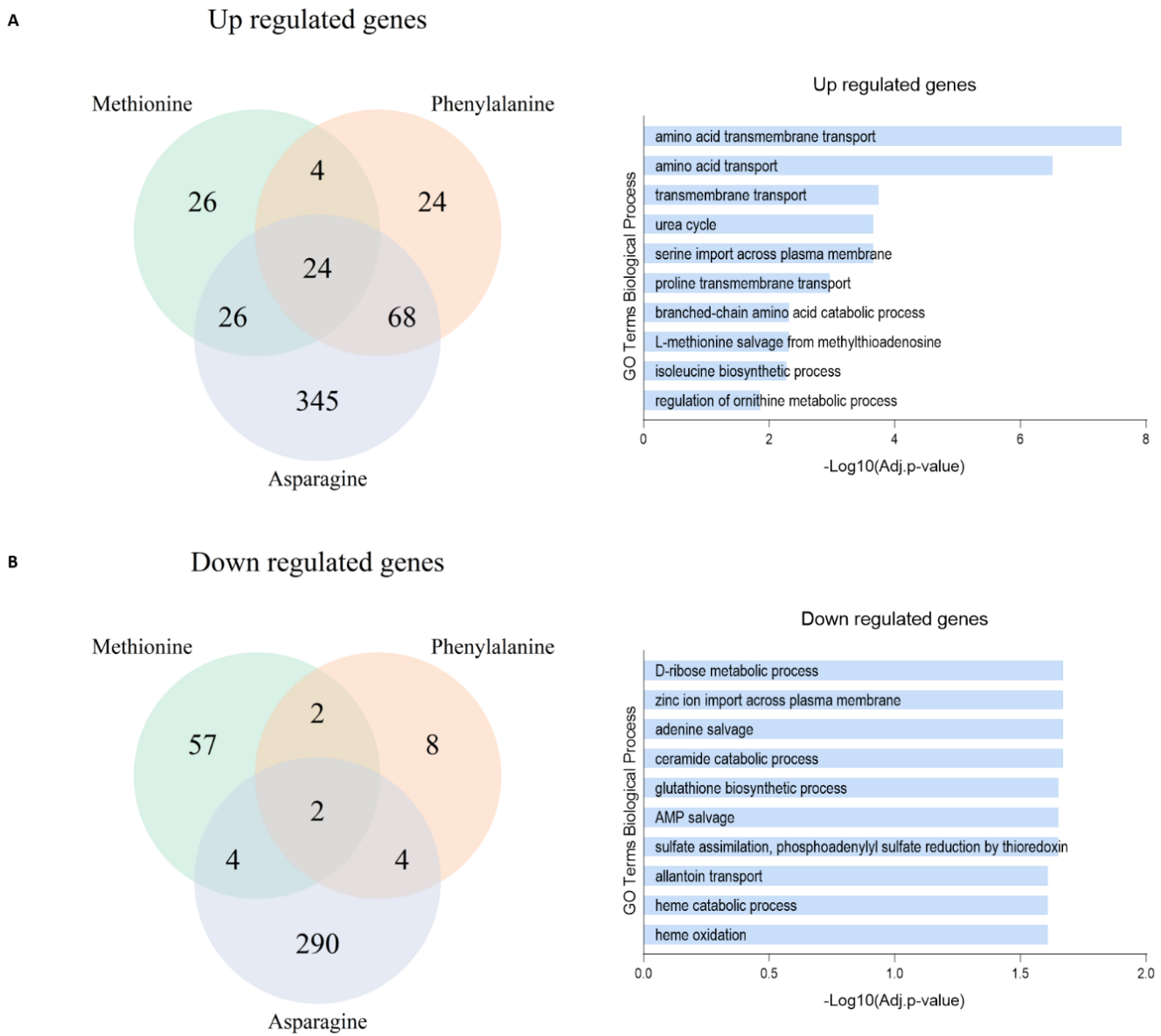


Figure 3. Global transcriptomic analysis. Venn diagrams showing significantly up regulated genes (A) and significantly down regulated genes (B) in methionine, phenylalanine and asparagine compared to ammonium-grown cultures. The selected genes have a fold change threshold of -1.5 and 1.5, and adjusted p-value <0.01. The overlaps show the numbers of genes found in common among all conditions. The top 10 GO Terms of biological process (BP) of the common genes are shown in bar plots. The categorising was done for 24 genes from the intersection of the most up regulated genes shown in panel A. In panel B the most down regulated genes used for the GO Terms analysis were 2 genes from the main intersection and 10 genes from the pairwise intersections.

Detailed response to each nitrogen source

Next, we examined the nitrogen-specific expression patterns by examining the individual response visualising the DESeq2 data in volcano plots that include fold change as well as adjusted p-values. The most differentially expressed genes were also categorized by GO Terms Biological Process (BP).

Effect of methionine

In methionine, 145 genes showed significant changes in expression compared to ammonium with 80 up-regulated and 65 down-regulated genes (Figure 4). Most highly up-regulated genes were in the BP category of BCAA biosynthetic process (e.g. *BAT1* and *BAT2*) and mostly related to amino acid metabolism. Amino acid transport and pathways involved in both the catabolism and biosynthesis of amino acids featured strongly. This reflects the use of methionine as a source of nitrogen for the biosynthesis of other amino acids. While some of the most highly expressed genes *MUP1, AGP1, BAP3* (methionine transporters), *ARO8, ARO9, BAT2, UGA1, ARO10, ADH5* (catabolic genes) are directly involved in methionine metabolism, others are not. The most up-regulated gene is *OPT2*, which encodes an oligopeptide transporter localised in the plasma membrane, peroxisome, and vacuole. Other important genes with the highest expression are *IRC7*, which encodes a beta-lyase involved in the production of thiols and desulfhydration of cysteine, and *SAM4* that maintains the methionine/AdoMet ratio in the cell.

The down-regulated genes in methionine were categorized in the sulfate assimilation pathway and methionine salvage pathway (MTA cycle) (*ADI1, SAM2, MET5, MET10, MET13, MET14, MET16, MET17* and *SUL2*). Other down-regulated genes not directly related to methionine metabolism are *CWP1* and *CWP2*, which are cell wall mannoproteins that stabilize the cell wall. One of the significant down-regulated GO Terms BP on methionine is mitochondrial electron transport, which includes the products of *SOD2, SUE1* and *GEP5* involved in respiration and *CYC1* and *COX13* involved in the electron transfer chain in mitochondria. Interestingly, *NDE1* encoding a

mitochondrial external NADH dehydrogenase that catalyses the oxidation of NADH in the cytoplasm, is repressed.

Effect of phenylalanine

When phenylalanine was used as the unique nitrogen source, there were 125 significantly up-regulated genes relative to ammonium, some of which are involved in the Ehrlich pathway. In the GO Terms annotation for phenylalanine, the most significant BP's include amino acid transport and stress related pathways such as glycogen biosynthesis (Figure 5B). The up-regulation of genes associated with nitrogen metabolism such as *AGP1, BAP3, TAT2, GNP1* (transport genes), *ARO9, ARO10, BAT2, ARO80, GDH2* (aroma and metabolic genes) was not unexpected based on prior work in *S. cerevisiae*. Nevertheless, some genes with high fold change are involved in carbon metabolism such as *MDH2, GDB1, XYL2, GPH1, GLC3, ATH1, GAL10, AMS1* and *CAT8.* On the other hand, the most down-regulated genes in phenylalanine (16 genes) are categorized in nucleotide and nucleoside metabolism, implying the low interconversion of hypoxanthine, purine, allantoin, adenine and pantothenate.

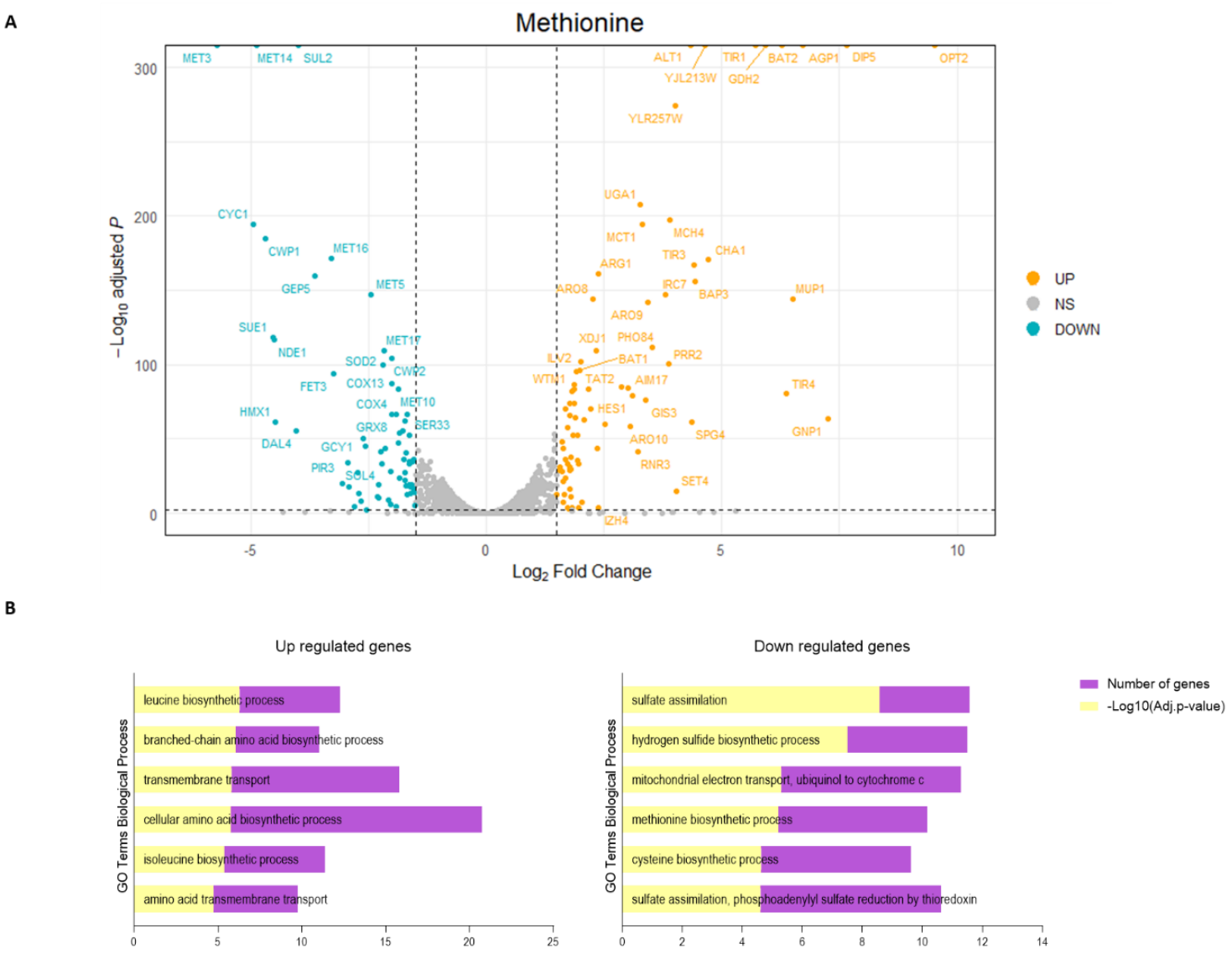
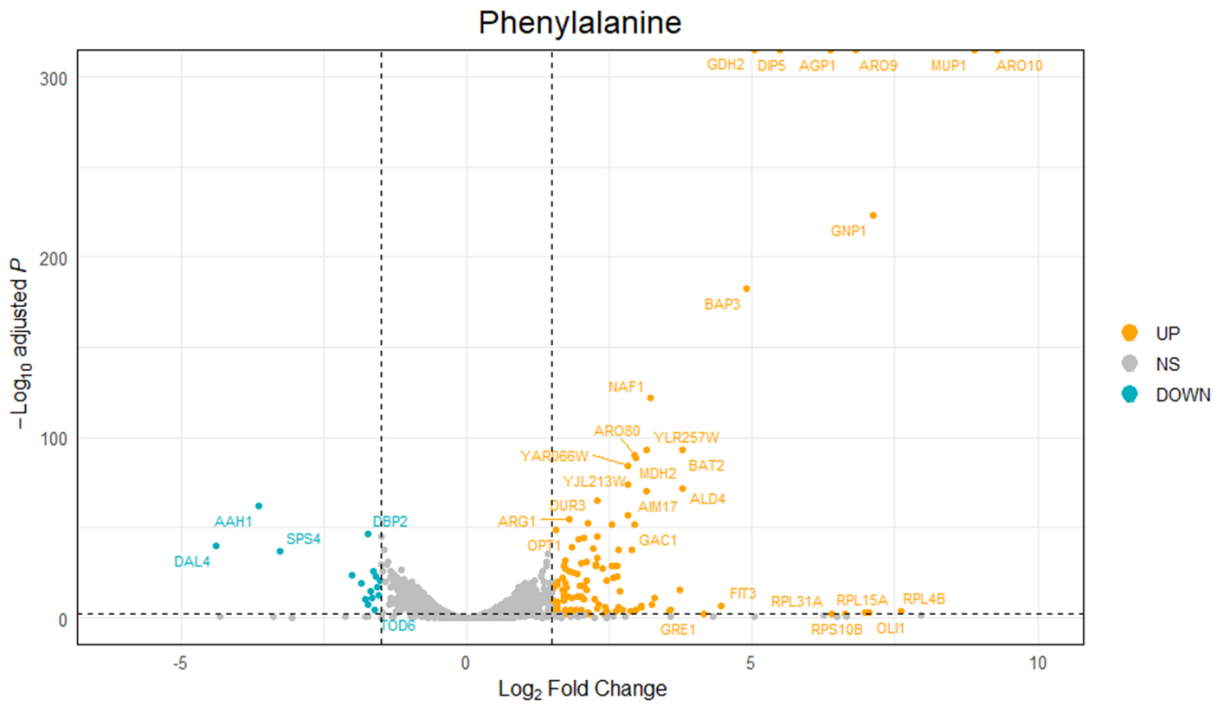


Figure 4. Gene expression in methionine. (A) Volcano plot representation of gene expression of *S. uvarum* under methionine conditions relative to ammonium. Down regulated genes are coloured in blue, up regulated genes are coloured in orange and non-significant (NS) genes are grey. The vertical black lines show a fold change (Log2FC) cut-off at -1.5 and 1.5. The horizontal black line shows the threshold of significance of adjusted p-value at 0.01. (B) Bar plots showing the GO Terms categories of biological process (BP) for the most up regulated and down regulated genes in methionine.

A



B

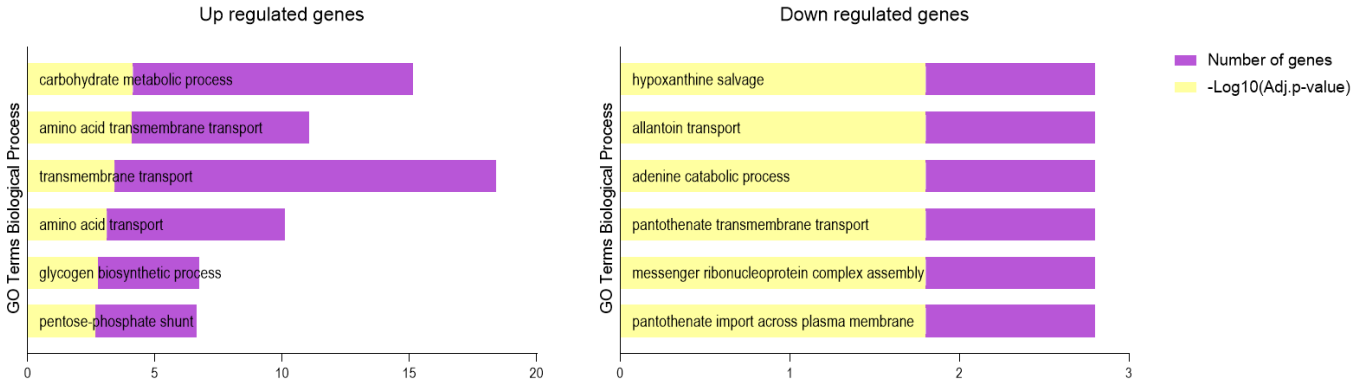


Figure 5. Gene expression in phenylalanine. (A) Volcano plot representation of gene expression of *S. uvarum* under phenylalanine conditions relative to ammonium. Down regulated genes are coloured in blue, up regulated genes are coloured in orange and non-significant (NS) genes are grey. The vertical black lines show a fold change (Log2FC) cut-off at -1.5 and 1.5. The horizontal black line shows the threshold of significance of adjusted p-value at 0.01. (B) Bar plots showing the GO Terms categories of biological process (BP) for the most up regulated and down regulated genes in phenylalanine.

Effect of asparagine

Asparagine showed the greatest change relative to ammonium and the highest number of genes (465 genes) among the amino acids tested. In general, the most enriched BPs were similar to phenylalanine condition, besides those, up-regulation of fatty acid beta-oxidation is an important biological process for energy obtention by the conversion of long chain fatty acids to short chain fatty acids. Looking at genes individually (Figure 6), similarly to phenylalanine and methionine, some of the most up-regulated genes are involved in nitrogen metabolism, however, some others (*MDH2, LEE1, PHO84*) are related to different pathways. For instance, *MDH2* is involved in carbon metabolism encoding a cytosolic malate dehydrogenase that produces oxaloacetate and reduces NAD to NADH in the cytosol. Moreover, the transcriptome analysis in asparagine revealed that the asparagine transporters *AGP1, DIP5, GNP1* are highly expressed, except from *GAP1*, thus efficient import of asparagine is expected. The asparagine transcriptome illustrated in Figure 6 showed 300 significantly down-regulated genes. The Biological Process GO Terms of down-regulated genes are related to biogenesis, assembly and maturation of ribosomes. This is an indication of poor quality of nutrients and slow growth in this condition.

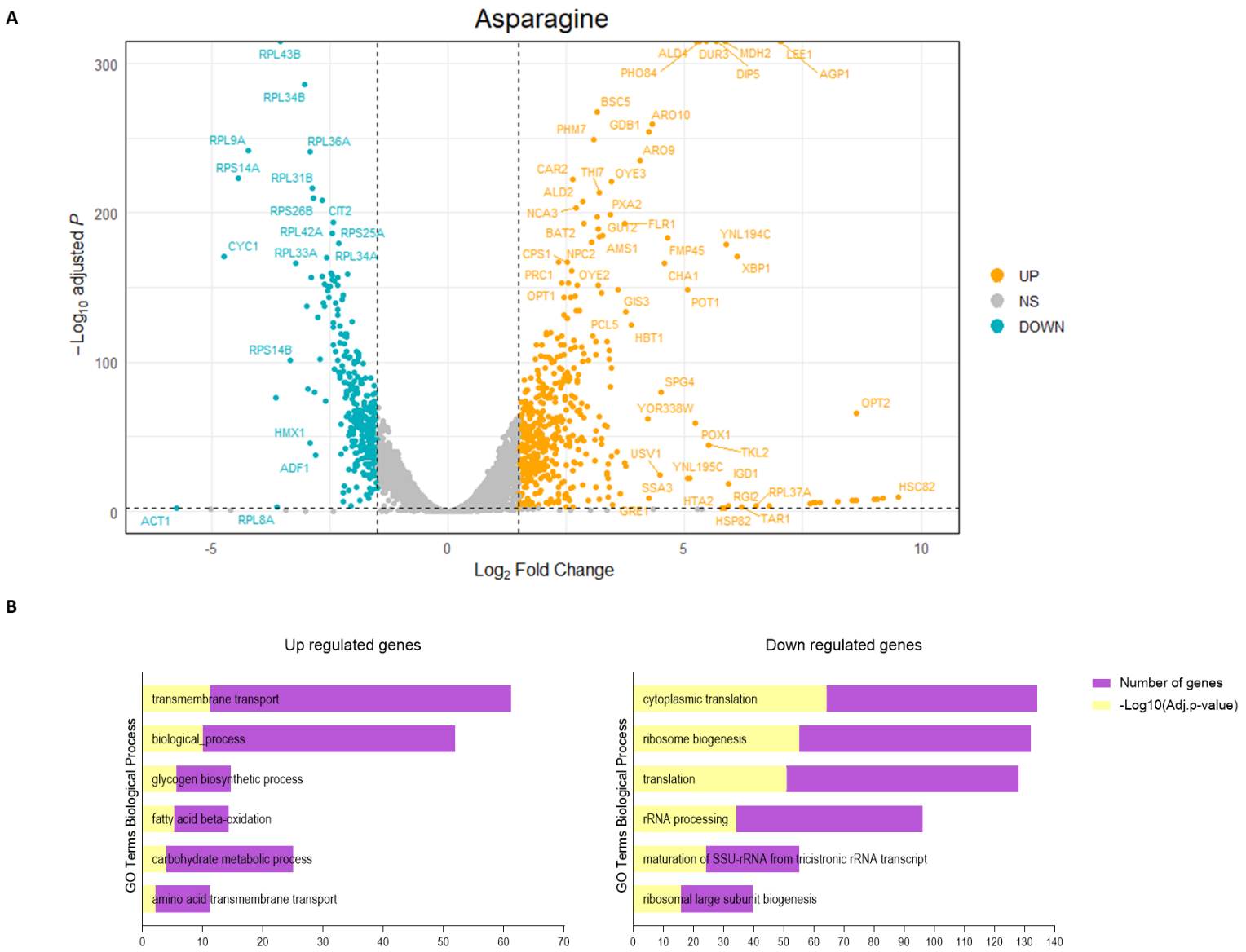


Figure 6. Gene expression in asparagine. (A) Volcano plot representation of gene expression of *S. uvarum* under asparagine conditions relative to ammonium. Down regulated genes are coloured in blue, up regulated genes are orange, and non-significant (NS) genes are grey. The vertical black lines show a fold change (Log2FC) cut-off at -1.5 and 1.5. The horizontal black line shows the threshold of significance of adjusted p-value at 0.01. (B) Bar plots showing the GO Terms categories of biological process (BP) for the most up regulated and down regulated genes in asparagine.

Differential expression of transcription factors and regulatory pathways in *S. uvarum*

The behavior of yeast strains can be understood by assessing the gene expression profile of regulation pathways as these represent the cell's response to the environment. Therefore, we examined the expression of transcription factors (TF) and other genes related to regulation pathways. None of the 24 upregulated genes in the three amino acids is a transcription factor indicating that the difference in regulation is not seen in the abundance of TF transcripts but in the target genes controlled by those factors. To analyze the contribution of specific regulation factors in detail, we constructed a heatmap illustrating the general differential expression of genes involved in different regulation mechanisms, namely NCR, the SPS sensor system, retrograde signaling (RTG), GAAC, and TOR (Figure 7). As a first outcome, most of the 27 genes shown the heatmap are up-regulated in all conditions compared to ammonium. Asparagine showed the highest expression of this cluster of genes and the most significant fold change was seen in GATA factors. Specially *GAT1* and *DAL80* are highly up-regulated in asparagine suggesting that NCR operates in *S. uvarum* similarly to *S. cerevisiae*. Likewise, *TOR1* and *MIG2,* which respond to nutrient availability and control cell growth, are overexpressed. Regarding methionine-grown cells, the regulation profile by GATA factors was similar to asparagine. Besides those, it was observed significant down-regulation of *MET28*, that together with *MET4,* participate in the regulation of sulfur metabolism. On the other hand, phenylalanine showed no differential expression of TFs relative to ammonium, except for *ARO80* that showed the highest expression as it is induced in the presence of aromatic amino acids.

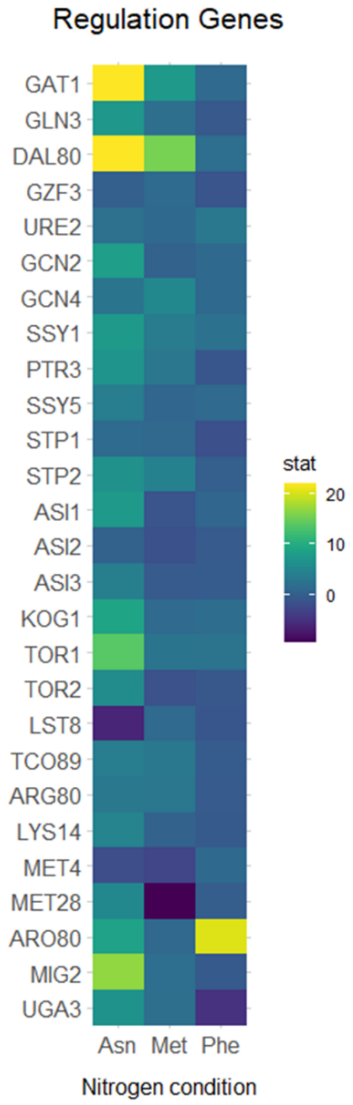


Figure 7. Influence of the nitrogen source on the expression of genes involved in regulation pathways. The genes are transcription factors and other enzymes that activate or repress target genes encoding nitrogen interconversion proteins. The genes belong to Ssy1-Ptr3-Ssy5 system (SPS), nitrogen catabolic repression (NCR), retrograde signalling pathway (RTG), the general control of amino acids (GAAC), and the TORC1 signalling pathway. The expression values are represented in colours with stat values obtained with the Wald test. Values >3 and <-3 are considered significant.

Transcriptional responses triggered by Ehrlich amino acids

The aromatic and branched amino acids are catabolised through the Ehrlich pathway. In this study, phenylalanine and methionine are referred as Ehrlich amino acids. The global gene expression on phenylalanine and methionine are plotted against each other to understand the relationship (Figure 8). In the upper right quadrant of the scatterplot the up-regulated genes *ARO10, ARO9, AGP1, MUP1, CHA1, GDH2, DIP5, GNP1, BAP3, BAT2* and *DUR3* are highly

expressed in both conditions, all involved in nitrogen metabolism and most of them regulated by NCR. The expression of *DUR3* specially, is highly sensitive to NCR (ElBerry et al., 1993). Moreover, some genes that are not directly involved in nitrogen interconversion pathways but are differentially expressed compared to ammonium were *MDH2* (carbon metabolites process), *MCT1* (fatty acids metabolic process) *MCH4* (carboxylic acids permease), and *YLR257W, YJL213W, YAR066W* of unknown function. Among those, *YJL213W* is a gene that was also reported to be expressed in *S. cerevisiae* in methionine conditions (Boer et al., 2007). Moreover, some interesting genes are shown in the up-left and down-right part of the plots, where the gene expression show a proportionally inverse relationship of both conditions. One of those genes is *NDE1* (mitochondrial NADH dehydrogenase) that is repressed in methionine but induced in phenylalanine. This opposite expression could indicate that *NDE1* is not influenced by the Ehrlich pathway factors.

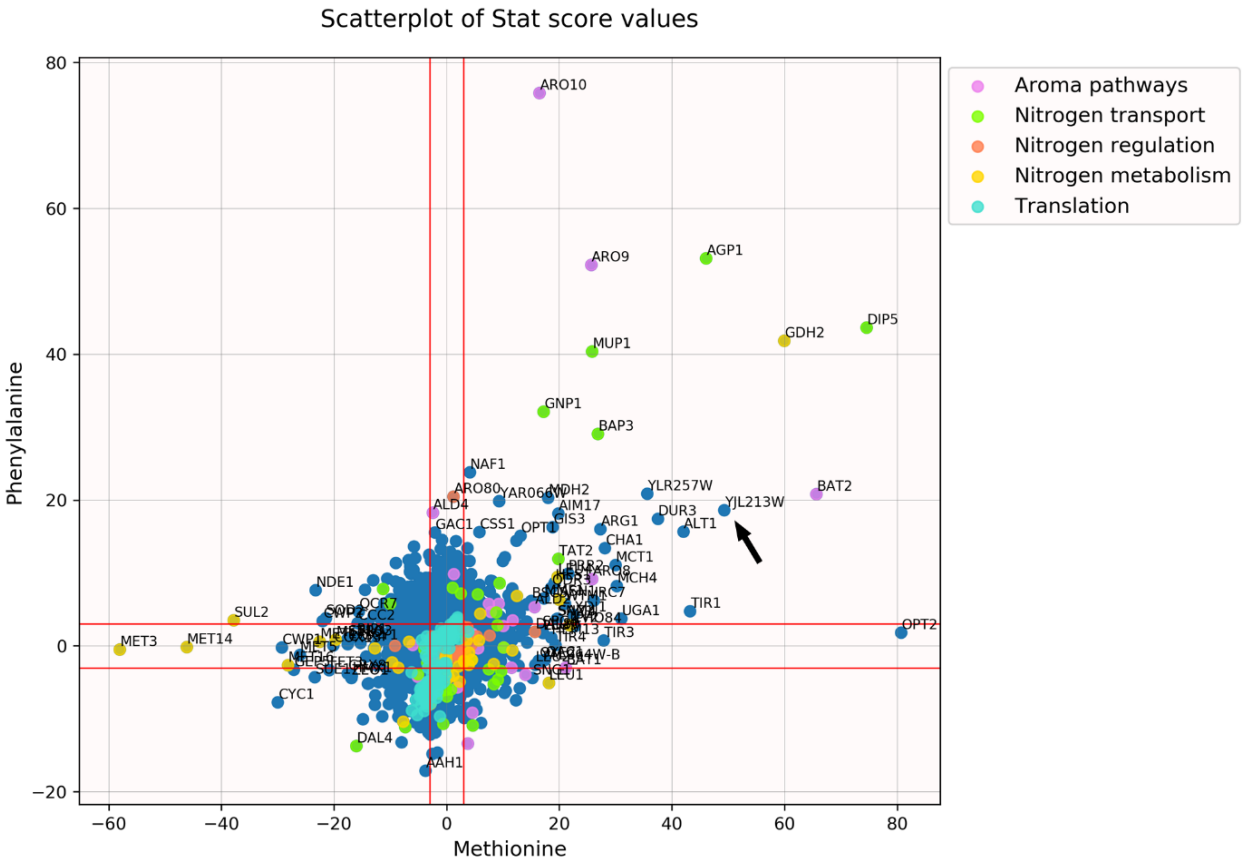


Figure 8. Relationship of gene expression between phenylalanine and methionine showing all the transcriptome genes. The dots represent individual genes of the two nitrogen conditions. The position of each gene on the horizontal and vertical axis indicates the relative gene expression in stat score values obtained with the Wald Statistic Test. The red lines show the threshold of significance in stat scores at 3 and -3. Important gene categories are represented in different colours. The black arrow indicates the *YJL213W* gene encoding a hypothetical protein with unknown function. Note that the scale of each axis is different.

Association of transcriptome and metabolome

To understand if there were changes in the transcriptome that might be expected to affect aroma production we associated the gene expression and metabolome produced in each condition. We focused on expression of genes involved in central carbon metabolism (CCM) and the aroma pathways (Figure 9). The metabolome, including CCM metabolites and volatile compounds, was also analysed at the end of batch fermentation (Figure 10) to determine the association with the transcriptome. The integral analysis was performed comparing data shown in Figure 9 and Figure 10. First, the fermentation metabolites mostly involved in the CCM showed particular profile depending on the nitrogen source. In general, ammonium had low production of CCM compounds while asparagine and phenylalanine showed increased production. In asparagine, high production of succinate, malate and α-ketoglutarate might be associated with up regulation of genes involved in carbohydrate metabolic process. The genes related to succinate and malate pathways (*SDHs, UGA2, MDH2*) are up-regulated in asparagine compared to the other conditions (Figure 9A). A positive correlation was found between the transcription of specific genes and high production of metabolites.

Turning now to the volatile metabolome (Figure 10B), generally, higher concentration of aroma compounds was observed in the Ehrlich amino acids. As expected, the production of some volatile compounds was increased in the precursor amino acid. Such is the case of 2-phenylethyl acetate and 2-phenylethanol in the presence of phenylalanine, and methionol and butanoic acid in the presence of methionine as sole nitrogen source. Methionine and phenylalanine also displayed high production of medium chain fatty acids (MCFA) and branched-chain fatty acids, probably as a consequence of the higher alcohols as precursors. In association to the gene expression, *ARO10, ARO9, ARO8, BAT1* and *BAT2* were significantly up-regulated in methionine and phenylalanine. Interestingly, *ARO80* (Figure 7) was also highly expressed in phenylalanine. In the case of asparagine, most of the volatile compounds showed decreased production compared to methionine and phenylalanine. The volatile metabolites with the highest production in asparagine were 2-methylpropanol and ethyl hexanoate, which also showed the highest concentration using ammonium as nitrogen source. This is an unexpected result since these two sources are not direct precursors of ethyl esters and also the growth profile is different between these sources.

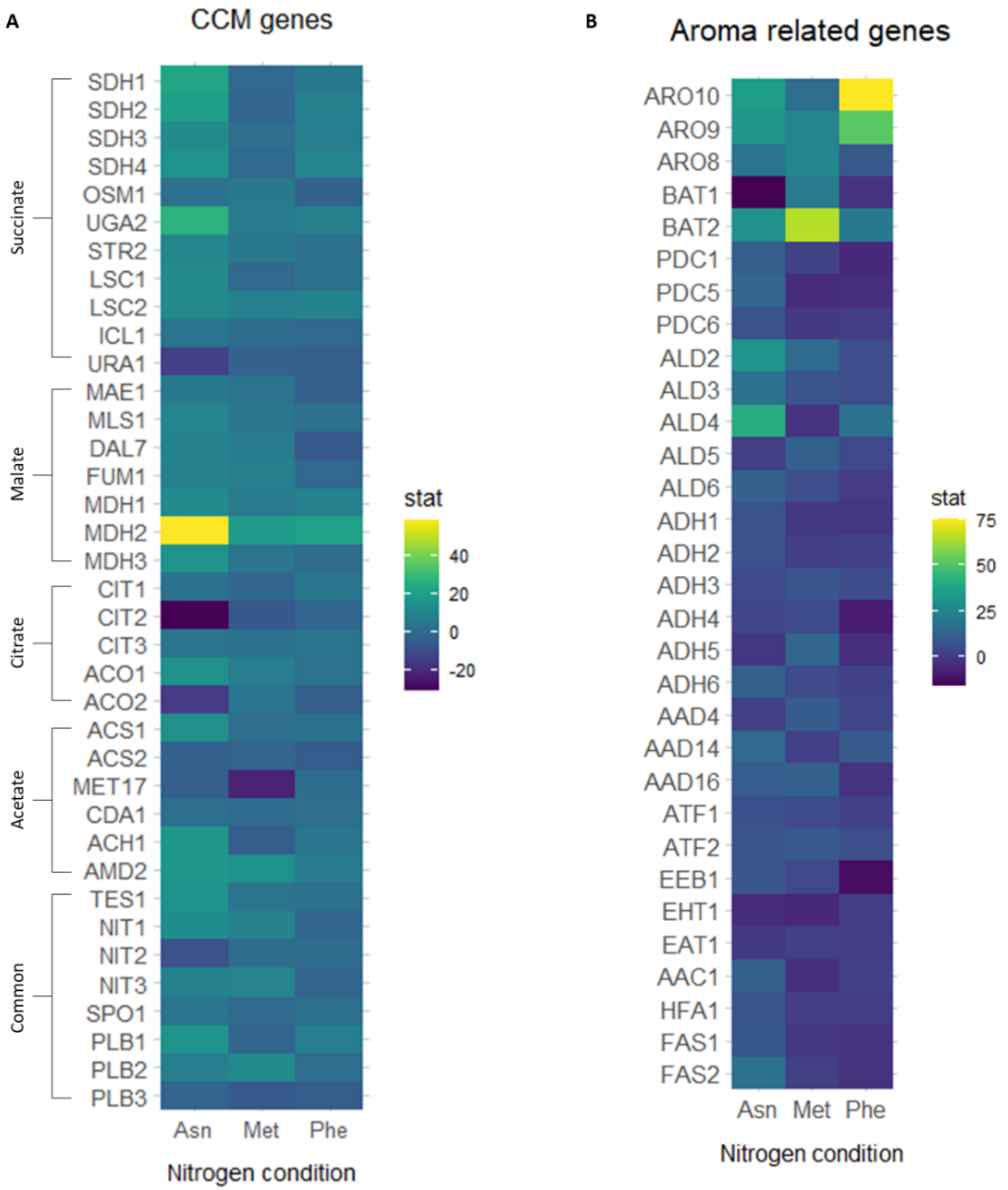


Figure 9. Influence of the nitrogen source on gene expression. Expression of genes involved in nitrogen metabolism and are target of transcriptional regulation genes. Fermentations done with asparagine (Asn), methionine (Met) and phenylalanine (Phe) as sole nitrogen sources. The genes encode enzymes involved in (A) central carbon metabolism (CCM) and (B) aroma production. The expression values are represented in colours with stat values obtained with the Wald test in each nitrogen condition relative to ammonium. Note that the Stat scales are different for each heatmap. Stat values >3 and <-3 are considered significant.

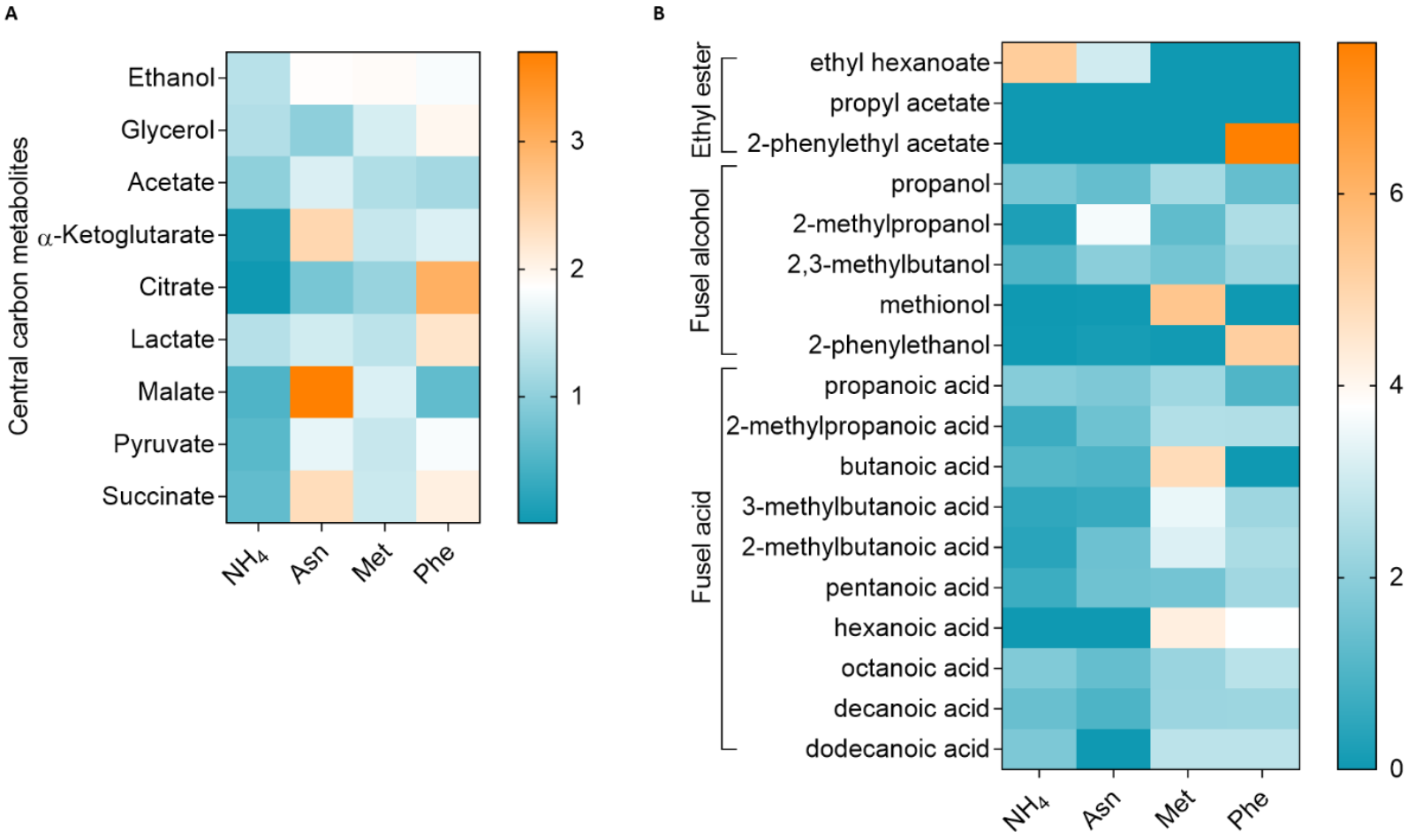


Figure 10. Metabolome of *S. uvarum* in different nitrogen sources. Differences on metabolite production of *S. uvarum* grown in ammonium (NH_4), asparagine (Asn), methionine (Met) and phenylalanine (Phe). Fermentations performed in minimal media containing glucose 20 g/L and nitrogen 1059 mgN/L at 20°C. (A) Final amount of central carbon metabolites (144 hours) produced during fermentation. (B) Final production of aroma volatiles. Values normalized to the mean of each compound produced among the nitrogen conditions. Only the volatiles that showed production are shown in the heatmap (18 compounds out of 34 measured).

Discovering the function of *YJL213W*

Most of the genes that showed very large changes in gene expression have already been annotated and the function is known. We were struck by one gene, *YJL213W*, that showed a very strong positive response to methionine but is unannotated and uncharacterised. Considering that this gene was also up-regulated in *S. cerevisiae* growing on methionine (Boer et al., 2007), it was possible that it may have a role in nitrogen metabolism. An *in silico* analysis of the predicted protein done with SWISS-MODEL server (Waterhouse et al., 2018) indicated that *YJL213W* is an amidohydrolase-related protein from the hydrolase superfamily, with hits to an imidazolonepropionase and the urease alpha subunit (Supplementary Figure 2). This suggests that the protein may be involved in nitrogen metabolites conversion, so we decided to inactivate it in *S. uvarum* to see whether there would be a phenotype. Interestingly, there are two highly similar copies of *YJL213W* in *S. uvarum* (90% DNA identity), while *S. cerevisiae* has only one gene. We generated *S. uvarum* mutants *Δyjl213w-1, Δyjl213w-2* and the double mutant *Δyjl213w-1 Δyjl213w-2* and evaluated growth with different amino acids as a sole nitrogen source. In spot assays on plates (Supplementary Figure 3), no difference was seen in any of the mutants but clear defects were evident in liquid medium (Figure 11). One of the single mutants was impaired but the double mutant *Δyjl213w-1,2* showed significant impaired growth compared to the wild type in ammonium, aspartate, glutamine, glutamate, valine and proline and a similar trend on other amino acids. The data shown in this paper, while preliminary, suggests that *YJL213W* is not an essential gene but it is involved in nitrogen metabolism. Metabolome analysis of the mutants is needed to understand if *YJL213W* has a role in the aroma production.

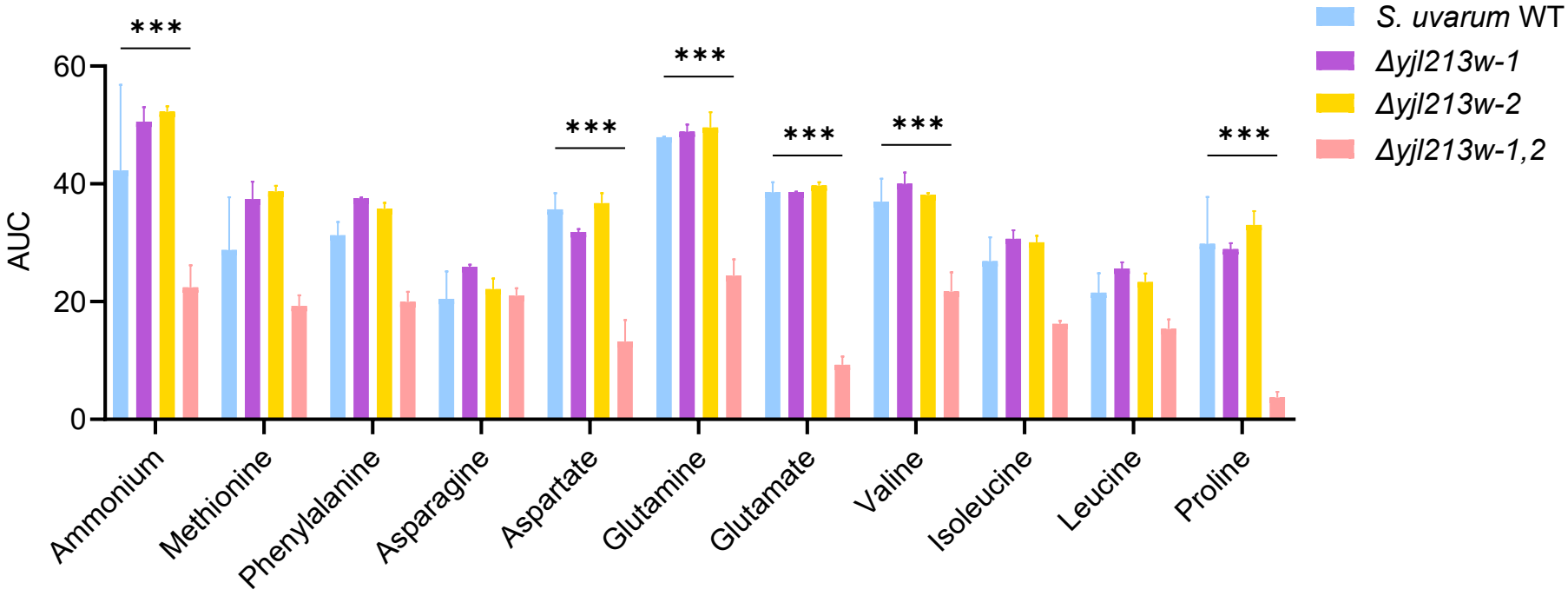


Figure 11. *YJL213W* has an important role on nitrogen metabolism in *S. uvarum*. The growth of *S. uvarum* wild type (WT), *S. uvarum Δyjl213w-1*, *S. uvarum Δyjl213w-2* and *S. uvarum Δyjl213w-1,2* was measured in minimal media individually supplemented with ammonium, methionine, phenylalanine, asparagine, aspartate, glutamine, glutamate, valine, isoleucine, leucine and proline at 1059 mgN/L. The *S. uvarum* WT was used as control for Two-way ANOVA significance test. Error bars were calculated from biological duplicates. Growth expressed as area under the curve (AUC). The difference in AUC is considered significant with p-value <0.001 (***).

DISCUSSION

The transcriptome analysis of *S. uvarum* revealed that the up-regulation of particular BPs and specific genes in response to different nitrogen conditions has a considerable effect on yeast metabolism and especially on the aroma profile. First, comparing gene expression of organic sources (amino acids) with the inorganic source (ammonium) we observed that the common response corresponds to the activation of the transport system and amino acid catabolic process. Conversely, the main differences of gene expression between methionine, phenylalanine and asparagine are explained, first by the nature of the nitrogen compound that is assimilated through different pathways, and second, by intermediates that might activate some sets of genes, depending on whether their promoters are controlled by the environmental conditions and pools of intermediate compounds. This is when the regulation pathways play a role to explain the yeast behaviour.

The data indicate that during assimilation of amino acids, the cell produced metabolites that are different from ammonium-grown cultures and those metabolites are inducers of particular catabolic genes. Moreover, most of the highly expressed genes in the tested amino acids are reported to be NCR regulated. This could indicate that the NCR mechanism is functioning in *S. uvarum* as it has been described in *S. cerevisiae*. For comparative analysis with *S. cerevisiae*, we used Boer et al., (2007) study as reference since that analysis was done in very similar conditions. There, for *S. cerevisiae,* it was reported that 23 genes were significantly up-regulated in non-preferred nitrogen sources (Met, Phe, Pro, Leu) differing from preferred sources (Asn and NH_4). From the 23 genes, 14 were established NCR targets, indicating that more than 50% of those genes are controlled by NCR (*ASN1, BAT2, CPS1, DAL1, DAL2, DCG1, DUR1,2, GDH2, GDH3, IDP1, MLS1, THR1, DAL5, DUR3, GAP1, MEP2, MEP3, OPT2, PTR2, DAL80, GAT1, YLR257W, YNL134C*). In our study in *S. uvarum*, we found that *BAT2, GDH2, DUR3, OPT2* and *YLR257W* are amongst the genes found to be up-regulated in non-preferred nitrogen sources. This is an indication of similar transcriptional control in *S. uvarum* and *S. cerevisiae* on sources that support intermediate/low growth. In non-preferred nitrogen sources, NCR is relieved leading to up-regulation of the same sets of genes in both species. Likewise, most of the non-nitrogen related genes that were up-regulated in each amino acid are regulated by NCR. Overall, the reasons for the expression of most of the genes shown here are found in studies done with *S. cerevisiae*, therefore, the transcriptome results

of *S. uvarum* indicated that these yeasts regulate nitrogen uptake and catabolism in a similar manner. However, there is the possibility that proteins activity and the affinity for transcriptional activation on promoters might be specific for *S. uvarum*, generating different phenotype.

Comparing the GO Terms annotation of methionine and phenylalanine, an interesting finding was the overexpression of genes involved in BCAA biosynthesis on methionine. Although methionine and phenylalanine are hydrophobic amino acids assimilated through the Ehrlich pathway, phenylalanine did not show this effect. This is possibly because the redistribution of nitrogen after transamination is less efficient in methionine, so the synthesis of BCAA starts *de novo* to supply the amino acids needed for biomass production. Shimizu et al., (2010) showed that *de novo* synthesis of BCAA contributes to NAD and NADP regeneration under hypoxia in *Aspergillus nidulans*; taking this study as reference of the fungi metabolism, it is possible to hypothesise that in methionine the BCAA biosynthesis pathways are induced in order to maintain the redox balance when oxygen is limiting. Moreover, the differences in gene expression observed on methionine and phenylalanine indicate that these amino acids produce different metabolic reorganization to adapt to the nitrogen source, as methionine is connected to the sulphur metabolism, while phenylalanine is mainly related to the pentose phosphate pathway. Additionally, *OPT2* was the most up-regulated gene in methionine (9.5-fold change). It is noteworthy that *OPT2* was also one of the 23 genes highly expressed in non-preferred nitrogen sources by *S. cerevisiae* (Boer et al., 2007) supporting the idea that Opt2 might have a role in non-preferred sources. Studies reported that Opt2 affects the glutathione redox homeostasis by transporting glutathione in different cell compartments, hence balancing glutathione homeostasis (Elbaz-Alon et al., 2014). This might explain the link to methionine metabolism due to the sulfur group of the amino acid. Opt2 is also involved in maintenance of vacuolar morphology and resistance to toxic agents detoxified by the vacuole (Aouida et al., 2009; Yamauchi et al., 2015). Additionally, the GO Terms annotations of phenylalanine showed up-regulation of BPs including amino acid transport and stress related pathways such as glycogen biosynthesis. Glycogen and trehalose biosynthesis are yeast responses that get active at the beginning of the stationary phase to resist different stress conditions such as high ethanol concentrations, heat shock and nutrient limitation (Parrou et al., 1997; Walkey et al., 2011). The activation of these processes in phenylalanine might be

due to stress caused by intracellular accumulation of some by-products of phenylalanine catabolism that could be toxic in high concentrations, such as phenylethyl acetate and phenylethanol.

Regarding the transcriptome data of *S. uvarum* in asparagine as sole nitrogen source, it is interesting that asparagine showed the most different transcriptome profile from ammonium (NH_4) since both NH_4 and asparagine are assimilated through the central nitrogen metabolism, differing from the Ehrlich amino acids. Also, the breakdown of asparagine produces aspartate and NH_4, so the transcriptomic response was expected to be more similar since NH_4 becomes a substrate in asparagine conditions. It was interesting to note that 13 of the genes highly expressed in asparagine in *S. uvarum* (*BAT2, CPS1, GDH2, GDH3, MLS1, THR1, DAL5, DUR3, DU1,2, OPT2, DAL80, GAT1, YLR257W*) were amongst the 23 genes reported by Boer et al., (2007) to be expressed in non-preferred nitrogen sources in *S. cerevisiae*. This identifies these genes as a signature of yeast growth on a non-preferred nitrogen source rather than being a response to specific amino acids. It also aligns with growth and nitrogen utilisation data: asparagine is a non-preferred amino acid in *S. uvarum* but preferred in *S. cerevisiae*. Finally, it shows that a metabolic transcriptomic signature is visible despite the growth rate caveats mentioned above and provides a degree of confidence in conclusions that are drawn from the asparagine transcriptome. Furthermore, the transcriptome analysis gave insights to explain why asparagine is not preferred by *S. uvarum* MTF3098. The transcriptome analysis revealed that the asparagine transporters *AGP1, DIP5, GNP1* are up-regulated, therefore efficient asparagine import is expected, although this would not be the case if these proteins display lower affinity for asparagine transport compared to what has been reported for *S. cerevisiae.* If transport is not the bottleneck for asparagine assimilation, it is possible that the issue lies with expression or activity of Asp1, the first enzyme involved in catabolism of asparagine in *S. uvarum*.

The GO Term annotations in asparagine showed down-regulation of ribosome processing process, explained by slow growth in this condition. In fast growing cells, the induced expression of ribosome genes is important for transcription and translation of metabolic genes used for nutrient assimilation, protein synthesis and production of essential compounds. Ribosome biogenesis is controlled by multiple mechanisms depending on nutrients availability and is an energetically expensive process for the cell. Ribosomal genes,

and a large set of genes of RNA metabolism and protein synthesis, were found to be repressed under environmental stress conditions (Gasch et al., 2000). This further supports the idea that ribosome synthetic genes are repressed in asparagine with the purpose of energy saving, as asparagine is not sufficient to support fast cell growth. In addition, *TOR1* up regulation in asparagine endorses this argument (Figure 7), since the main link between nutrients availability and ribosomes production is done by the TORC1 signalling complex (Wullschleger et al., 2006).

Association of the aromatic intensity of *S. uvarum* with the differential expression of orthologous genes involved in nitrogen metabolism among *Saccharomyces* yeast, is an interesting trait to study (Stribny et al., 2016). The transcriptome data revealed the expression of important nitrogen related genes in *S. uvarum* that directly contribute to the aroma profile. An important CCM metabolite highly produced by *S. uvarum* is succinate, which showed increased production on phenylalanine and asparagine; this is in agreement with studies reporting influence of the nitrogen source on the production of succinate (Camarasa et al., 2003).

The final production of volatile compounds can be associated to the transcriptome since the final aroma profile is a result of gene expression during fermentation. The production of some volatile compounds is correlated to the availability of the amino acid as it serves as precursor of biosynthetic reaction. Additionally, it was observed that the production of volatile compounds is linked to a set of up-regulated genes such as Ehrlich pathway genes, *ADH* and *ALD* genes (upregulated in methionine and phenylalanine). Our findings suggest that the up-regulation of specific (single) genes has an important effect on the volatile metabolome of *S. uvarum*. An example of this is the significant up regulation of *ARO80* in phenylalanine. This finding is in agreement with Tapia et al., (2022) who showed that Aro80 efficiently activates the expression of *ARO9* and *ARO10* in *S. uvarum,* being an important trait for phenylethanol and phenylethyl acetate production. Moreover, the genes involved in aromatic amino acid catabolism are regulated by interaction between Aro80 and GATA activators (Lee and Hahn, 2013), and these factors require the presence of Aro80 to bind to the promoters of aromatic genes. The induction of *ARO10* and *ARO9* is higher when aromatic amino acids are present to induce the activity of *ARO80,* but even in the absence of these amino acids, Aro80 and

Gat1/Gln3 are enough to induce the expression of *ARO* genes, such is the case of asparagine condition in this study.

Growth rate has an effect on the nitrogen regulation pathways, however, the effect at the level of aroma production seems to be unrelated to growth. Contrary to what we observed for the production of CCM metabolites, we suggest that growth rate might not have an influence in the aroma production since the aroma profile in ammonium and asparagine is similar, even if the growth pattern is very different. Therefore, the volatile metabolites production is mainly attributed to the nature of the nitrogen compound and the assimilation pathways. Nevertheless, these data must be interpreted with caution because here we are just relying on the comparison of two nitrogen conditions. Further experiments are needed to elucidate the effect of growth rate on the aroma profile.

Taken together, these findings and the comparative analysis done with *S. cerevisiae*, suggest that the most likely cause of the difference of phenotype between the species is the effect of catabolic intermediates on the regulatory pathways and expression of aroma genes. Especially, the presence/efficiency of important proteins involved in the direct assimilation of the nitrogen compounds. This paper established a baseline on how the nitrogen source affects the transcriptome in relation to the production of aroma compounds. Future research should focus on temperature as a factor influencing the aroma production in *S. uvarum* to understand if this has a role in the gene expression. For this purpose, the same approach of integral analysis of volatile metabolome and transcriptome might be used. Furthermore, to completely elucidate the differences between the species that explain the unique aroma profile of *S. uvarum*, a detailed gene by gene comparison with *S. cerevisiae* could be beneficial.

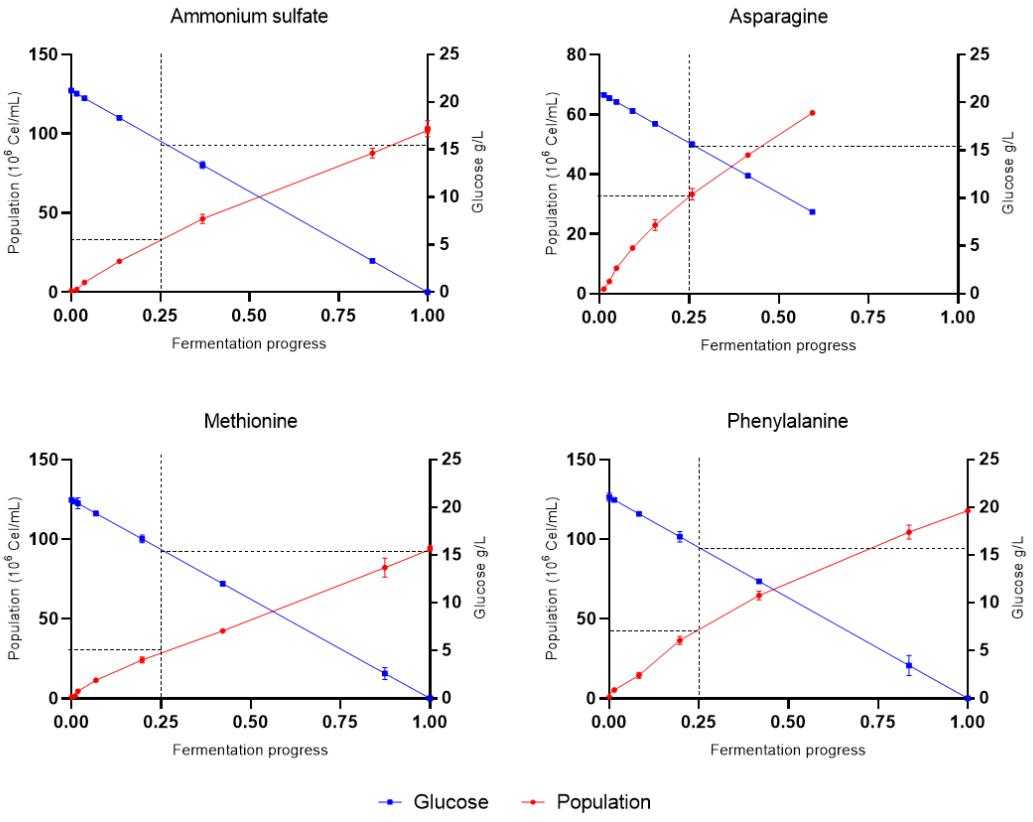
Supplementary information The online version contains supplementary material available at DOI:https://doi.org/10.5281/zenodo.6678834

Conflict of Interest The authors have no relevant financial or non-financial interest to disclose.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Oligonucleotides used for deletion of *YJL213W* in *S. uvarum.*

PRIMER NAME	SEQUENCE (5'-3')
CRISPR_T1_SuYJL213W-1_F	CGTCTCGTGCCACTACGGTCAAGT
CRISPR_T1_SuYJL213W-1_R	AAACACTTGACCGTAGTGGCACGA
RF_SuYJL213W-1_F	TTGATGTAAACGAAGGGATGAACAAGTCCCTAGCCGATCTGGTAGCGCCATGGCGGCAGAGATGAATGGC
RF_SuYJL213W-1_R	CAATGAACCCTGGTTTAATCTGACCTAGTTTGTTGTCCAAGCCATTCATCTCTGCCGCCATGGCGCTACC
Diag_SuYJL213W-1_F	TTGCTGCTGTGAATAACGAAACTTGACC
Diag_SuYJL213W-1_R	GCAACAAAGTGAAACTCTAGACCTAAAACG
CRISPR_T1_SuYJL213W-2_F	CGTCCAAGTTGGTGTTGTAGCAGA
CRISPR_T1_SuYJL213W-2_R	AAACTCTGCTACAACACCAACTTG
RF_SuYJL213W-2_F	TAACACCAATTTGATAGATGTAGTAAGCGGTACTACTTGCCCTGAGGCTTAcatcGGGTCCTCTATATGG
RF_SuYJL213W-2_R	CTGAACCTTTCCTCTGATACGGAACTCTTGTGTTTGATAACCATATAGAGGACCCgatgTAAGCCTCAGG
Diag_SuYJL213W-2_F	atgacAACTAAAGATATTGATGTAAACGAAGGAATGA
Diag_SuYJL213W-2_R	AAAAATTCTTCCATCTTTCATCACAAGCAAC
BSA-clon-R	TACACGCGTTTGTACAGAAAAAAAAGAAAAATTTGA

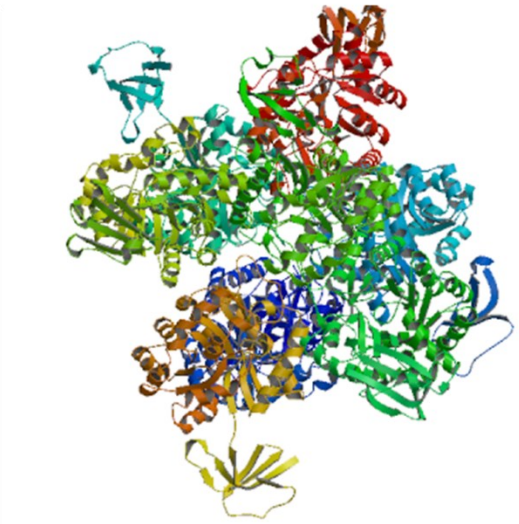


Supplementary Figure 1. Sugar and biomass conditions at sampling point. Population normalized to the maximum population (Pop/Pop_{max}) on the left Y axis and Glucose (g/L) on the right Y axis, as a function of Fermentation progress for fermentations done with *S. uvarum* in Minimal Medium for 48 hours. The dash black lines are showing the sampling points. Mean values were calculated from biological triplicates.

Template Results

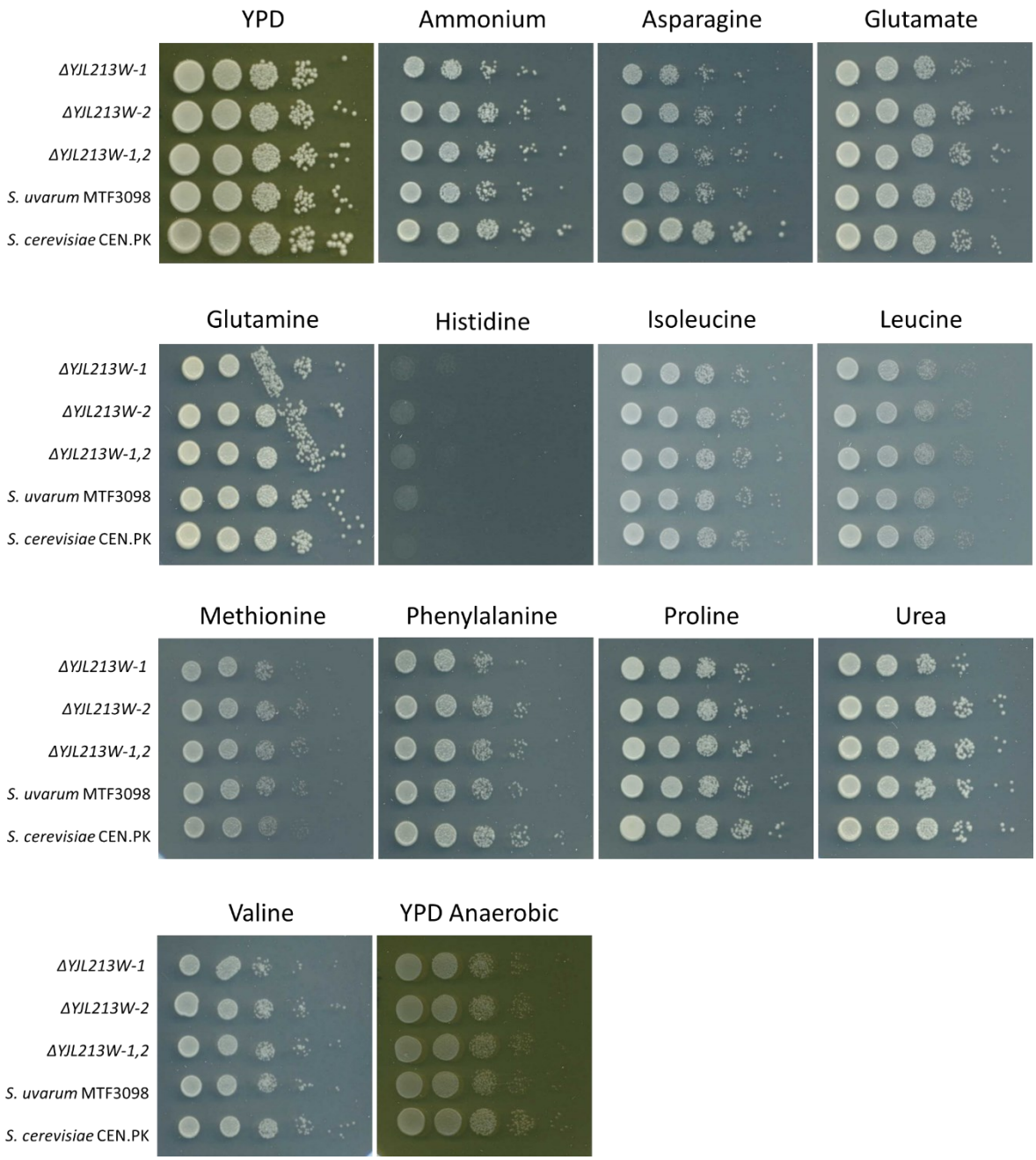
A total of 1756 templates were found to match the target sequence. This list was filtered by a heuristic down to 50. The top templates are:

Template	Sequence Identity	Biounit Oligo State	Description
3mkv.1	42.74	homo-octamer	PUTATIVE AMIDOHYDROLASE Crystal structure of amidohydrolase eaj56179
3feq.1	40.90	homo-octamer	PUTATIVE AMIDOHYDROLASE Crystal structure of uncharacterized protein eah89906
3mkv.1	45.25	homo-octamer	PUTATIVE AMIDOHYDROLASE Crystal structure of amidohydrolase eaj56179
3n2c.1	40.90	homo-octamer	PROLIDASE Crystal structure of prolidase eah89906 complexed with n-methylphosphonate-l-proline
3n2c.1	45.22	homo-octamer	PROLIDASE Crystal structure of prolidase eah89906 complexed with n-methylphosphonate-l-proline



Predicted protein structure

Supplementary Figure 2. SWISS MODEL of YJL213W protein structure and domain classification. Genomic sequence obtained from *S. uvarum de novo* assembly (This study).



Supplementary Figure 3. Spot assays of growth for 48 hours. *S. cerevisiae* CENPK113-7D, *S. uvarum* MTF3098 (wild type), the mutants *S. uvarum Δyjl213w-1, S. uvarum Δyjl213w-2* and the double mutant *S. uvarum Δyjl213w-1,2* evaluated in plates containing minimal media (2% agar) supplemented with different nitrogen compounds as a sole (except from YPD plates used as control).

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Chapter 3

The evolution and role of the periplasmic asparaginase Asp3 in yeast

Angela Coral-Medina[1,2], Darren A. Fenton[1,3], Javier Varela[1,4], Pavel V. Baranov[3], Carole Camarasa[2], John P. Morrissey[1]*

[1]School of Microbiology, University College Cork, Ireland; [2]SPO, Univ Montpellier, INRAE, Institut Agro, Montpellier, France; [3]School of Biochemistry and Cell Biology, University College Cork, Ireland [4]Current address: Metabolic engineering department, CarboCode GmbH, Konstanz, Germany.

*Corresponding author: j.morrissey@ucc.ie

ABSTRACT

The study of nitrogen assimilation in yeast is of interest from genetic, evolutionary, and biotechnological perspectives. Over the course of evolution, yeasts have developed sophisticated control mechanisms to regulate nitrogen metabolism, with domesticated lineages sometimes displaying particular specialisation. The focus of this study was on assimilation of asparagine, which is a significant nutritional source for some alcoholic fermentations. We were particularly interested in *ASP3*, which encodes a periplasmic asparaginase and that was proposed to have been acquired relatively recently in *S. cerevisiae* by horizontal gene transfer. We examined 1680 *S. cerevisiae* genome assemblies to evaluate the distribution and evolutionary trajectory of *ASP3.* Our findings suggest an alternative hypothesis that *ASP3* is an ancient *Saccharomyces* gene that has generally been lost over the course of evolution but has been retained in certain fermentative environments. As asparagine is the major nitrogen source in apple juice, we explored whether the presence of *ASP3* would confer a growth advantage. Interestingly, we found that although *ASP3* enhances growth when asparagine is the sole nitrogen source, the same effect is not seen in apple juice. These data indicate that growth in pure culture may not reflect the original selective environment for *ASP3*$^{+}$ strains and highlight the role that complex regulation may play in optimising nitrogen assimilation in yeasts.

INTRODUCTION

Although the evolution of yeast for alcoholic fermentation preceded human civilisation by millions of years, it is the trait that underpinned and drove the domestication of multiple species in the *Saccharomyces* genus (Dashko, Zhou, Compagno, & Piškur, 2014). Species of *Saccharomyces* were used in traditional food and beverage fermentations and were then domesticated for particular applications like beer, bread, wine, cider and more recently

bioethanol fermentations (Almeida et al., 2014; Fleet, 2008; Gallone et al., 2016; Verstrepen et al., 2001). There are a range of different types of genetic change that underpin the phenotypic changes and subsequent adaptation to human-related (anthropogenic) environments (Gallone et al., 2018; Giannakou, Cotterrell, & Delneri, 2020). Within a genome, point mutations alter the structure/function of proteins and influence gene expression, while larger variations result in chromosomal rearrangements, segmental duplication, and variation of gene copy number. Introduction of new genes via horizontal gene transfer (HGT) is also an important means by which strains acquire new phenotypes; for example, the capacity to use particular nutrients (Galeote et al., 2010; Hall, Brachat, & Dietrich, 2005; Novo et al., 2009). Interspecific hybridization has also emerged as a critical means of adapting to anthropogenic environments, with multiple instances from the *Saccharomyces* genus (Alsammar & Delneri, 2020) and from other budding yeasts (Solieri et al., 2021).

Nitrogen is a limiting factor in alcoholic beverage fermentation and there are several known examples where domesticated *Saccharomyces* strains evolved more efficient nitrogen assimilation (Almeida et al., 2014; Becerra-Rodríguez, Marsit, & Galeote, 2020; Marsit & Dequin, 2015). Depending on the particular beverage, the ammonium salts, amino acids and small peptides that are assimilated by yeast are present in the fermentation substrates in different proportions. While free ammonium is a very good nitrogen source for yeast, it is generally only available in small amounts and most nitrogen is provided by free amino acids that are transported into the cell and then further catabolised. Amino acids that are more readily catabolised and sustain higher growth rates are typically used first by yeast and are considered preferred nitrogen sources (Boer et al., 2007; Ljungdahl & Daignan-Fornier, 2012). *S. cerevisiae* has sophisticated regulatory pathways to control and optimise acquisition of nitrogen, namely, TORC-regulated Nitrogen Catabolite Repression (NCR) (Magasanik & Kaiser, 2002) and the SPS-sensor system (Ljungdahl, 2009), which detect the nitrogen

conditions in the medium and deploy signalling and transcriptional responses to induce or repress genes involved in the transport and metabolism of certain nitrogen compounds (Zhang, Du, Zhou, & Chen, 2018).

In *S. cerevisiae,* asparagine, which is important for yeasts used for fermented food and beverages, is a preferred nitrogen source (Boer et al., 2007). Although, it is not a significant amino acid in grapes, it is found in other fruits and in beer wort. A study of the amino acid composition of fruit juices revealed that the quantity of asparagine in apple juice is 100 times higher than in grape juice (Dizy, Martín-Alvarez, Cabezudo, & Carmen Polo, 1992). Likewise, Ma et al., (2018) analysed the amino acid content of 13 samples of apple juice from different cultivars, and reported that asparagine, aspartate and glutamine are the prominent amino acids in most of the apple juices, with asparagine being present at the highest concentration. Asparagine is a yeast nutrient in wort (Ferreira & Guido, 2018), proved for the first time by Garza-Ulloa et al., (1986) who reported that yeast consumes asparagine during beer fermentation. The canonical pathway (Figure 1) for asparagine assimilation starts with transport into the cell by one of the general amino acid transporters Agp1, Gnp1, Dip5 or Gap1 (Bianchi, Ruiz, & Poolman, 2019), all of which are transcriptionally regulated by NCR and the SPS sensor system (Ljungdahl & Daignan-Fornier, 2012). Within the cytosol, a constitutively expressed asparaginase I (Asp1) hydrolyses the amide group of the side chain of asparagine generating one molecule each of aspartate and assimilable ammonia, which is the major nitrogen intermediate for amino acid biosynthesis (Dunlop et al., 1978 (Sinclair, Warner, & Bonthron, 1994)). Some strains of *S. cerevisiae* have a second asparaginase encoded by *ASP3*. Asp3 is located in the periplasmic space and can degrade asparagine outside the cell (Dunlop & Roon, 1975; Dunlop, Roon, & Even, 1976; Jones & Mortimer, 1973). In this case, the resulting ammonium enters the cell via one of the dedicated Mep transporters and the aspartate is transported by the general transporters Gap1 and Dip5, which are under NCR and SPS

control, or a dedicated transporter named Agp3. *ASP3* is also controlled by NCR and its expression is upregulated in the absence of rich nitrogen sources to facilitate the utilization of extracellular asparagine (Oliveira, Martins, Carvajal, & Bon, 2003; Scherens, Feller, Vierendeels, Messenguy, & Dubois, 2006).

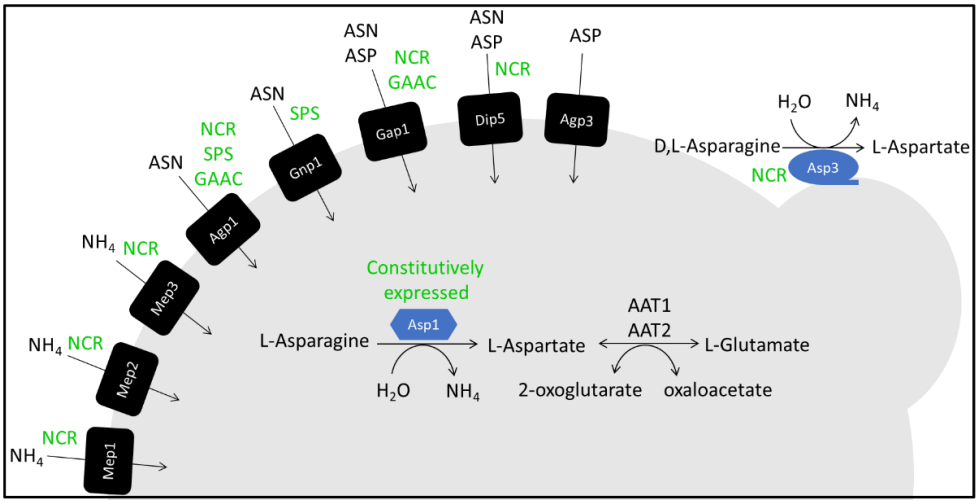

Figure 1. Asparagine assimilation in *S. cerevisiae*. Asparagine (ASN) is imported into the cell through the transporters Agp1, Gnp1, Gap1 and Dip5; Aspartate (ASP) by Gap1, Dip5 and Agp3; and Ammonium (NH_4) by the specific transporters Mep1, Mep2 and Mep3. Extracellular hydrolysis of asparagine is done by Asp3 localised in the cell wall, while Asp1 hydrolyses asparagine in the cytosol. Both enzymes catalyse the same reaction, producing aspartate and NH_4. Intracellular aspartate is converted to glutamate and oxaloacetate, which are easily utilised by the cell through the central nitrogen metabolism. Nitrogen Catabolite Repression (NCR), the SPS-sensor system (SPS) and General Amino Acid Control (GAAC) are the regulation mechanisms of each protein shown in green while other genes are constitutively expressed.

ASP1 is universally present in the *Saccharomycotina* (budding yeast) whereas *ASP3* has only been reported in some *S. cerevisiae* strains and rarely elsewhere. The low prevalence and presence of a homologous gene in *Wickerhamomyces anomalus* led to the hypothesis that the presence of *ASP3* in *S. cerevisiae* could be due to HGT from *W. anomalus* in biotechnological environments, where the yeasts co-occurred (League, Slot, & Rokas, 2012). In this regard, the fact that apple juice is rich in asparagine, and both *S. cerevisiae* and *W. anomalus* are frequently associated with spontaneous cider fermentations, points to an environment where such a transfer could take place. The rationale would be that the presence of Asp3

offered strains a growth advantage, whereby they would more efficiently assimilate asparagine during fermentation.

We are interested in how yeasts adapt to domestic niches and decided to investigate the role and history of Asp3. The large number of genome sequences now available provide an opportunity to revisit the hypothesis of HGT. We were also curious to see whether Asp3 would be found in *Saccharomyces* species other than *S. cerevisiae*, especially in *Saccharomyces uvarum*, which is commonly used in cider fermentations. In this study, we first determined whether the HGT hypothesis is supported by more recent genomic data. The next question was whether the presence of *ASP3* confers an advantage for yeast growth and nitrogen assimilation. And finally, we investigated whether fermentations of natural matrices (apple juice) support the idea that *ASP3* confers a selective advantage. Our analysis led to the conclusion that *ASP3* is most likely to be an ancestral gene that was not recently acquired but in fact has been lost in the majority of *S. cerevisiae* strains. We used heterologous expression of *ASP3* in *S. uvarum* to establish that *ASP3* does confer a growth advantage when asparagine was the sole nitrogen source, but unexpectedly this did not translate into better growth in apple juice where asparagine is the nitrogen source. The seemingly contradictory findings that retention of *ASP3* is enriched in strains used for beer or cider fermentations but the gene appears not confer a growth benefit in this environment, will be discussed.

MATERIALS AND METHODS

Yeast strains and growth

The *Saccharomyces* strains isolated from different sources used for this study are listed in Table 1. Species identity of all strains was confirmed by sequencing the D1-D2 region of the 25S rRNA gene. (Kurtzman & Robnett, 1997). The cryopreserved yeast strains were plated in Yeast Peptone Dextrose (YPD) agar and incubated during 48h. For growth experiments, pre-

cultures were grown in YPD broth at 28°C for 16 hours with shaking (180 rpm), then transferred to YNB media without ammonium and amino acids for 4 hours at 28°C to exhaust the yeast nitrogen reserves. The cells were washed with sterile 0.9% NaCl to remove all nitrogen residues and then resuspended in minimal synthetic medium (MM) (glucose 20g/L, KH_2PO_4 3g/L, $MgSO_4.7H_2O$ 0.5 g/L, vitamin mix, and trace elements in concentrations adapted from Verduyn media (Verduyn, Postma, Scheffers, & Van Dijken, 1992). The nitrogen source was provided as required for each experiment. This cells suspension was further used to inoculate fermenters or microtitre plates. For microtitre plate fermentations, precultures treated as described above diluted in sterile water to A_{600} 0.5 and then used to then inoculate 200 µL fresh media at A_{600nm} 0.05 (1:10 dilution). The 96 flat wells microplate was incubated at 20°C in the microplate reader CLARIOStar®^{Plus} (BMG LABTECH, Germany). A_{600nm} was measured for 60 cycles of 1 hour (24 flashes/cycle) with continual double orbital shaking (400 rpm) between measurements. This experiment was done with a minimum of three biological replicates of each strain in each media, using one blank per media. *S. cerevisiae* CENPK113-7D was used as control in all the microtitre plates.

Fermentation conditions

Batch fermentations were performed in 330 mL fermenters containing 250 mL of medium, equipped with airlocks to prevent the entry of air but allowing CO_2 release during fermentation. The fermenters were aerated for 20 minutes before inoculation to balance the dissolved oxygen content with the air. Yeasts were inoculated at $5x10^5$ cells/mL (~0.04 A_{600} for *S. uvarum* and ~0.05 A_{600} for *S. cerevisiae*). The fermentations were conducted with continuous magnetic stirring (230 rpm) at 20°C. Fermentations were performed in biological triplicates of each strain. The fermentations were monitored by CO_2 release, weighting the fermenters to track record of weight loss. The CO_2 production rate (g/L/h) was calculated by polynomial smoothing of the last eight values of CO_2 production. The sampling points were determined depending on the growth and consisted of two types of

sampling depending on the methods to be performed afterwards. For frequent sampling, 1.5 mL of medium was taken to monitor the cell population with Coulter counter (BECKMAN®) and spectrophotometer (A_{600}). The samples (6 mL) destined for HPLC and GCMS analysis were taken at the beginning of the exponential phase, at 40 g/L of CO_2 produced in the case of cider, and at the end of fermentation. Then, the samples were centrifuged to remove cells pellet and the supernatant was stored at -20°C for further HPLC and GCMS analysis. The cider fermentations were carried out on microfiltered apple juice A843 (Guillevic apple variety from Bretagne, France) provided by the Institut Francais des Productions Cidricoles (IFPC). The apple juice (pH 3.52) contained ~108 g/L of sugar at a ratio of glucose to fructose of 3:7, and ~127 mg/L of nitrogen, made up of asparagine (95 mgN/L), aspartate 10 mgN/L), traces of other amino acids, and NH_4 (0.6 mgN/L). Conditions and measurements were as described for batch fermentations except that the experiments were performed at 16°C.

Construction of overexpression strains

To express *ASP3* in *S. uvarum,* the gene was amplified by PCR from *S. cerevisiae* CENPK113-7D using Q5 polymerase (New England Biolabs (NEB) Inc., MA, USA) and cloned into the pGREG-505-TEF1 plasmid (Varela et al., 2017) via Gibson Assembly (New England Biolabs (NEB) Inc., MA, USA). All primers and plasmids are listed in Tables. The assembly reaction was transformed into *E. coli* and then plated on LB medium supplemented with ampicillin 100 µg/mL. The resulting plasmid pGREG-505-TEF1-ASP3 was recovered and verified with HindIII enzymatic digestion (NEB) and sequencing (Eurofins Genomics, Germany). pGREG-505-TEF1-ASP3 was transformed into *S. uvarum* MTF3098 using the LiAC/SS carrier DNA/PEG procedure (Gietz & Schiestl, 2007) with selection on YPD agar supplemented with G418 200 µg/mL. *ASP3* was also integrated into the *HO* locus of *S. uvarum* MTF3098. The integration fragment, called GREG-ASP3 (1843 bp), consisting of the TEF1 promoter, *ASP3* coding sequence and *CYC1* terminator, was PCR amplified from pGREG-505-TEF1-ASP3 with primers

containing homology regions to the *HO* gene. A plasmid was constructed to introduce a CRISPR Cas9-mediated double stranded break at the *HO* locus. For this, oligonucleotides containing the target *HO* sequence were cloned into pUDP002-HH using Golden Gate assembly as previously described (Rajkumar, Varela, Juergens, Daran, & Morrissey, 2019). Finally, the CRIPSR plasmid targeting *HO* and the GREG-ASP3 repair fragment were co-transformed into *S. uvarum* MTF3098. The cells were plated on YPD supplemented with hygromycin 200 ng/µL and the correct integration of *ASP3* at the *HO* locus was confirmed via PCR with diagnostic primers.

Analytical methods

Free amino acid content in the media was measured by cation exchange chromatography (Biochrom 30, Biochrom, Cambridge, UK) as previously described (Crépin, Nidelet, Sanchez, Dequin, & Camarasa, 2012). The measurement of glucose, fructose, ethanol and other central carbon metabolites was done with High-Performance Liquid Chromatography (HPLC) on Phenomenex Rezex ROA column (HPLC HP1100 Infinity, Agilent Technologies) as described by Rollero et al., 2015. Volatile compounds were measured by gas chromatography/mass spectrometry (GC-MS) as described in detail by Rollero et al., 2015. In brief, a liquid extraction of supernatants to recover higher alcohols, fatty acids, acetate esters and ethyl esters was performed with dichloromethane CH_2Cl_2, the organic phase was recovered, dried, and concentrated with nitrogen gas before injection onto a Hewlett Packard 6890 gas chromatograph (Agilent Technologies, Santa Clara, California, USA) equipped with a CTC Combi PAL Autosampler AOC-5000 (Shimadzu, Columbia, USA), coupled to a HP 5973 mass spectrometry detector (HP, now Agilent Technologies, Santa Clara, California, USA).

Bioinformatic and molecular analysis of *ASP3*

ASP3 sequences were identified *in silico* with BLASTP and TBLASTN using the *S. cerevisiae* S288c *ASP3* sequence (Agarwala et al., 2016). Strains without annotations were annotated using Augustus (Stanke & Morgenstern, 2005).

The Peter et al., (2018) tree and other phylogenetic trees were displayed using Iroki (https://www.iroki.net/) with custom tables to highlight taxa (Moore, Harrison, McAllister, Polson, & Eric Wommack, 2020). Phylogenetic trees for Asp3 protein sequences were generated with RAxML-NG (Kozlov, Darriba, Flouri, Morel, & Stamatakis, 2019) using the model parameters LG+G4. Synteny analysis at species level was performed using the MCSCAN pipeline as part of JCVI utility libraries (Tang et al., 2008). Synteny within *S. cerevisiae* was performed with a series of python scripts incorporating BLAST, annotations and contigs/scaffold information. PCR was used to detect the presence of *ASP3* in *Saccharomyces* species. PCR amplification was done using the OneTaq master mix (New England Biolabs (NEB) Inc., MA, USA) with conserved interspecies primers designed to amplify *ASP3* in different yeast species, including *S. cerevisiae, Z. parabailii* and *W. anomalus* (Supplementary Table 1). The thermocycling conditions were as follows: an initial enzyme activation of 1 minute at 95°C, followed by 30 cycles of denaturation for 15 seconds at 95°C, annealing at 52°C for 15 seconds and extension for 90 seconds at 65°C, with a final elongation step of 5 minutes at 68°C. The evaluated strains included 10 *S. cerevisiae* strains, 5 *S. uvarum* strains and other *Saccharomyces* species such as *S. kudriavzevii, S. arboricola, S. eubayanus, S. jurei, S. paradoxus* and *S. mikatae* (Supplementary Table 2). The expected *ASP3* fragments 1089 bp long were detected via 1% gel electrophoresis.

Statistical analysis

The statistical analysis of the growth kinetics data was performed using R Studio software, version 1.3.1093 (RStudio Team., 2020) with GrowthCurver package (Sprouffske & Wagner, 2016). The reported growth kinetics are expressed in the metric area under the curve (AUC) that integrates information from the logistic parameters: lag phase length, growth rate, final time and maximum biomass produced (Supplementary Figure 6). The AUC metric is a measure of growth that has been used in previous nitrogen preference studies (Su et al., 2020). For the fermentation data, the graphs

and statistical tests were performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California, USA).

Table 1. Yeast strains used in growth studies. Additional strains are listed in supplementary material.

Yeast	Strain	Geographical origin	Environment	*ASP3* status
S. cerevisiae	S288c	-	Laboratory	4 copies
S. cerevisiae	CEN.PK113-7D	-	Laboratory	1 copy
S. cerevisiae	EC1118	Champagne, France	Wine	Absent
S. uvarum	BMV58/MTF3098	Valencia, Spain	Wine	Absent
S. uvarum	CBS7001/OS24	Avila, Spain	Insect Mesophylax adopersus	Absent
S. uvarum	OS472	Marlborough, New Zealand	End of Sauvignon Blanc Wine fermentation	Absent
S. uvarum	CBS395	The Netherlands	Juice of Ribes nigrum	Absent
S. uvarum	CLIB1050	France	Cider must	Absent
S. uvarum	CLIB393	Japan	Cider brewery	Absent
S. uvarum	CLIB501	Normandie, France	Industrial sweet cider	Absent
S. uvarum	ΔHO::ASP3	-	-	1 copy

RESULTS

ASP3, an ancestral gene of *S. cerevisiae* lost throughout evolution

To study the distribution of *ASP3* within *S. cerevisiae* strains, a database with 1680 strains was constructed using publicly available genome assemblies. Using BLASTP, we detected the presence of at least one copy of *ASP3* in the genomes of 117 strains (~7%). These data are mapped onto the previously published *S. cerevisiae* phylogenetic tree (Peter et al., 2018), which allowed us to investigate the distribution of *ASP3*-containing strains (Figure 2A; for expanded tree including all *S. cerevisiae* strains see Supplementary Figure 1). For many of these strains, the isolation source is known allowing us to ask whether the distribution of *ASP3* showed any patterns (Supplementary Table 3). Although there is not an absolute correlation between the presence of *ASP3* and the environmental or geographical origin of the strains, *ASP3* is predominantly found in strains associated with the bioethanol & food industry, brewing & cider making, and research laboratories. It is interesting to see that *ASP3* is largely absent in strains isolated from wine environments.

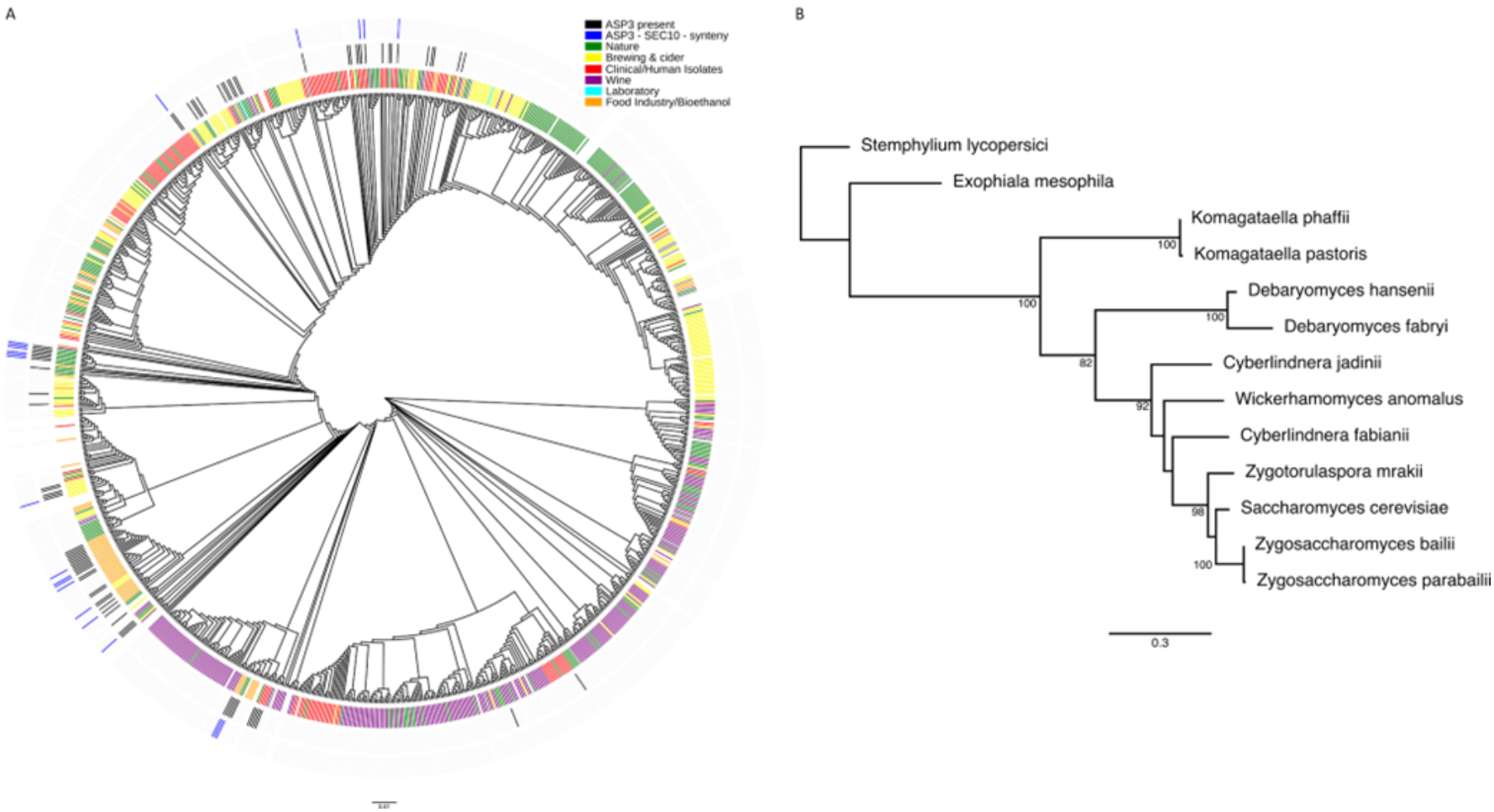

Figure 2. *ASP3* phylogeny in budding yeasts. (A) The distribution of *ASP3* in *S. cerevisiae* is shown as black bars in the second ring that is superimposed in a multigenic phylogenetic tree of 963 *S. cerevisiae* strains (from Peter *et al*. 2018). The isolation source of the strains is highlighted in the first ring using the colours: green (nature), yellow (brewing & cider), red (clinical/human isolates), purple (wine), light blue (laboratory) and orange (bioethanol & food industry). The outer blue bars mark strains with conserved *ASP3* synteny based on identification of a conserved ASP3-MAS1-PUS5-SEC10 locus. (B) The evolutionary relationship of *ASP3* in budding yeast was determined by aligning protein sequences and drawing a phylogenetic tree. Only bootstrap values above 70% are displayed on the phylogenetic tree.

To examine the relationship of the *S. cerevisiae ASP3* genes to each other and to *ASP3* in other ascomycetes, we first interrogated databases to identify homologous genes using a combination of BLASTP and TBLASTN to ensure that both annotated and unannotated genes were found. In this way, we identified *ASP3* homologues in *Stemphylium lycopersici, Exophiala mesophile, Komagataella phaffi, Komagataella pastoris, Debaryomyces hansenii, Debaryomyces fabryi, Cyberlindera jadinii, Cyberlindera fabianii, Zygotorulaspora mrakii, Zygosaccharomyces parabailii,* and *Wickerhamomyces anomalus* (where it was already known). We aligned the protein sequences and drew phylogenetic trees to see the relationships. This revealed that all the *S. cerevisiae* Asp3 sequences are more similar to each other than any other Asp3 and thus derive from a common ancestor (Figure 2B). It is also seen that the distribution of Asp3 amongst yeast species largely follows the pattern that would be expected in a yeast species phylogenetic tree and *S. cerevisiae* Asp3 is most related to the Asp3 found in *Zygosaccharomyces/Zygotorulaspora* species. Next, we examined the contig/scaffold containing *ASP3* of each *S. cerevisiae* strain to determine whether *ASP3* is present in a conserved locus. It was only possible to perform this synteny analysis in forty strains because in most of the genome assemblies, *ASP3* is the sole protein-coding gene on that contig/scaffold. In all forty cases, however, *ASP3* is adjacent to *MAS1, PUS5* and *SEC10 (ASP3-MAS1-PUS5-SEC10*) showing that *ASP3* is located within a conserved location that we refer to as the *ASP3-SEC10* locus (Supplementary Figure 2). We added these to the phylogenetic tree (Figure 2A, outer ring) and, as with *ASP3* itself, this syntenic locus is distributed across the tree. We also analysed the *ASP3* synteny in other yeast species but failed to find any relationship between *ASP3* location in any of these species (Supplementary Figure 2). Considering the conserved synteny and the sequence similarity of the protein sequences, it is concluded that *ASP3* is ancestral in *S. cerevisiae* and, while its prevalence seems enriched in strains from certain human-associated environments, the gene was not recently acquired by horizontal gene transfer. The low number of available sequences and lack of synteny

preclude any strong conclusion for other yeast species, but the phylogenetic pattern is most consistent with ancestral carriage of *ASP3* in the *Saccharomycotina* and subsequent widespread loss.

The apparent enrichment of *ASP3* in fermentation-associated though not wine isolates of *S. cerevisiae* led us to assess whether the gene might be present, but unidentified, in other species of *Saccharomyces*. Using primers that would amplify *ASP3* in different yeast species (Supplementary Table 1), we performed PCR on genomic DNA from eight different *Saccharomyces* species (21 strains) and found that *ASP3* was only present in the control *S. cerevisiae*. Specifically for *S. uvarum*, as well as the five strains examined by PCR, we performed *in silico* analysis of a further twenty-four strains, and again failed to detect *ASP3* (Supplementary method). Although, it must be acknowledged that a much larger set of strains for each species would need to be examined to rule out carriage at a low frequency, the data indicate that *S. uvarum* appears not to encode *ASP3*.

Asparagine consumption profile is different in *S. cerevisiae* and *S. uvarum* species

One explanation for retention of *ASP3* in some strains of *S. cerevisiae* would be that Asp3 confers an advantage for growth using asparagine as a nitrogen source. To investigate this, we performed growth tests on a set of ten *Saccharomyces* strains using asparagine, aspartate or ammonium as the sole nitrogen source (Figure 3). The strains came from wine or cider sources and included three isolates of *S. cerevisiae* (carrying 1, 4 or no copies of *ASP3*) and seven *S. uvarum* isolates (all *ASP3* negative). All strains encode Asp1 and so can use asparagine as a sole nitrogen source, but the questions were whether the presence of Asp3 would confer an advantage, whether there was a difference between the species, and whether the origin of the strain had an effect. For *S. cerevisiae*, it is seen that asparagine and ammonium are preferred to aspartate but the strain lacking Asp3 (EC1118) did not stand out as growing less than the other strains on asparagine, thus there is no evident advantage to *ASP3* strains. In the case of *S. uvarum*, a uniform pattern was

not seen regarding preferences for nitrogen sources. For example, the strain CLIB1050 grew poorly on aspartate but well on asparagine and ammonium, whereas the strain OS24 grew poorly on asparagine but well on the other two nitrogen sources. Overall, with the exception of the slow growing CLIB1050 (on aspartate), the growth of both species was similar on aspartate and ammonium, but a difference was seen on asparagine. Here, the *S. uvarum* strains bifurcated with two strains growing similarly to *S. cerevisiae* and five strains growing much less well. The striking observation was that the two better growing *S. uvarum* strains were isolated from cider fermentations and four of the strains that grew poorly specifically on asparagine, came from the wine environment. Although numbers of strains included in this analysis are low, it could indicate that cider strains of *S. uvarum* have adapted to use asparagine more efficiently, regardless of the presence or absence of *ASP3*.

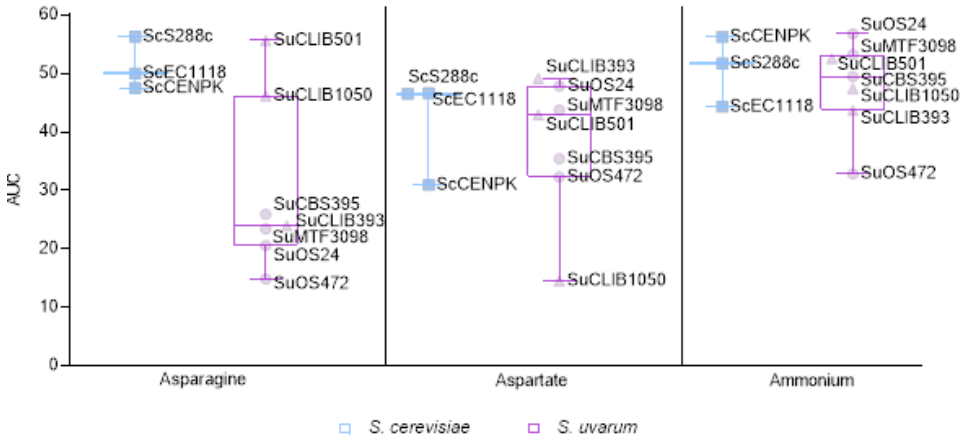

Figure 3. Preference of the nitrogen source by *S. cerevisiae* and *S. uvarum* strains. Growth of three *S. cerevisiae* and seven *S. uvarum* strains on minimal media with asparagine, aspartate, or ammonium as sole nitrogen source (1059 mgN/L) was measured. Growth is represented by the parameter "area under the curve (AUC)". Data are presented as boxplots and represent the mean of three replicates. The origin of the *S. uvarum* strains are represented with circles (wine) or triangles (cider). In *S. cerevisiae* strains the origin is not discriminated. Growth measured from biological triplicates of each strain.

To further investigate the differences in the capacity of *S. cerevisiae* and *S. uvarum* to use asparagine as sole nitrogen source, two strains were selected

for deeper analysis. *S. cerevisiae* CEN.PK113-7D (*ASP1, ASP3*) and *S. uvarum* MTF3098 (*ASP*1) were used for the batch fermentations in MM containing either asparagine or ammonium as the sole nitrogen source (Figure 4). Fermentations were monitored for 48 hours measuring the population size, CO_2 production rate, glucose consumption and ethanol production. No substantial differences were seen in the fermentation profile of *S. cerevisiae* in asparagine and ammonium (Figure 4B) and *S. uvarum* in ammonium (Figure 4A). In those cases, the cells reached stationary phase and the maximum rate of CO_2 production after approximately 32 hours of fermentation, glucose was depleted after 36 hours, and the ethanol production was approximately 6 g/L. It is noted that *S. uvarum* does achieve a larger population size than *S. cerevisiae* on ammonium. In contrast, for the fermentations with *S. uvarum* in asparagine, after 48 hours the cells were still in an active growth phase and 40% of glucose remained in the media. The maximum specific growth rate (μ_{max}) for *S. uvarum* in asparagine was 0.10 h^{-1}, whereas it was 0.27 h^{-1} for *S. cerevisiae*. In *S. cerevisiae* a similar population size was reached on both nitrogen sources but for *S. uvarum*, after 48h, the population size on asparagine was substantially lower than that on ammonium. These data confirm that asparagine is not a preferred carbon source for *S. uvarum.*

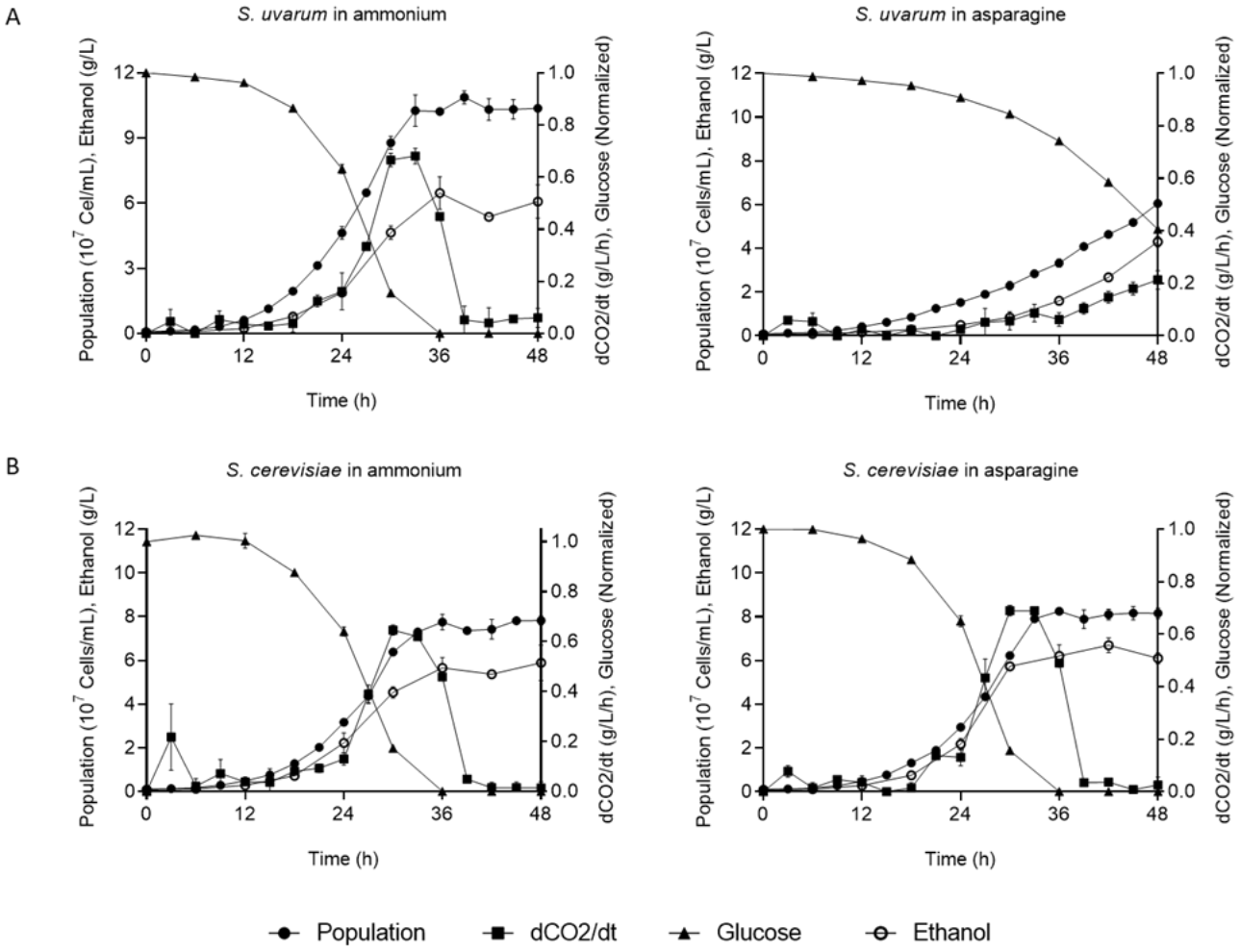

Figure 4. Fermentation profile of *S. uvarum* and *S. cerevisiae* strains on ammonium and asparagine. Fermentations of *S. uvarum* MTF3098 (panel A) and *S. cerevisiae* CENPK113-7D (panel B) were carried out at 30°C, on minimal medium with excess of asparagine (right) and ammonium (left) as sole nitrogen sources (1059 mgN/L) and glucose (20 g/L) for 48h. Each graph shows the progress of glucose consumption normalized to 1 (initial), ethanol production expressed in g/L, the CO_2 production rate expressed in g/L/h and the cell population size expressed in cells/mL. The error bars were calculated from biological triplicates.

The role of *ASP3* in growth and the production of secondary metabolites in *S. uvarum*

We next wanted to investigate whether *ASP3* could confer an advantage for yeast growth with asparagine as a nitrogen source as that could provide an explanation for selective retention in some lineages. As it was apparent from the data in Figure 3 that strain background would be a confounding aspect, we decided to work in a congenic background and heterologously expressed *ASP3* in *S. uvarum* MTF3098, which lacks an endogenous copy. We evaluated the growth of three *S. uvarum* strains: the wild type (WT), a strain with *ASP3* on a plasmid and a strain carrying *ASP3* in the genome (constitutive promoter in both cases). Fermentations were conducted in MM with asparagine or ammonium provided in excess. Initial experiments found that growth in ammonium was similar for the three strains but, in asparagine, the strains expressing *ASP3* grew more efficiently (Supplementary Figure 3). This led us to perform a more comprehensive in-depth comparison of the fermentation profiles in MM containing 100 mgN/L asparagine of the wild-type strain with *S. uvarum::ASP3*, which carries a single constitutively expressed copy of *ASP3* in its genome (Figure 5). Comparison of four key growth parameters showed that *S. uvarum::ASP3* (dashed line) consumed glucose faster, accumulated ethanol and glycerol more rapidly, and achieved maximum population size earlier than the wild-type (WT) *S. uvarum* (Figure 5A). The faster fermentation of *S. uvarum::ASP3* was also evident in the production of other central carbon metabolites and volatile compounds, where concentrations of propanol, 2-methylpropanol and 2,3-methylbutanol were higher in *S. uvarum*::*ASP3* after 30 hours but both strains showed similar profile of metabolites produced at the end of fermentation (Supplementary Figure 4). We also followed how the concentrations of asparagine, aspartate and ammonium varied over the fermentation (Figure 5B). Both strains consumed the same amount of asparagine within 60 hours, but they displayed very different pattern of consumption. *S. uvarum* WT showed low consumption of asparagine during

the first 30 hours of fermentation, with low accumulation of aspartate in the medium (below 20 mg/L). In contrast, during the first 18 hours of the *S. uvarum*::*ASP3* fermentations there was rapid consumption of asparagine and a higher accumulation of aspartate (30 mg/L). Ammonium concentration was constant during the 60 hours for both strains, so the cells consumed it immediately as it was not accumulated in the media.

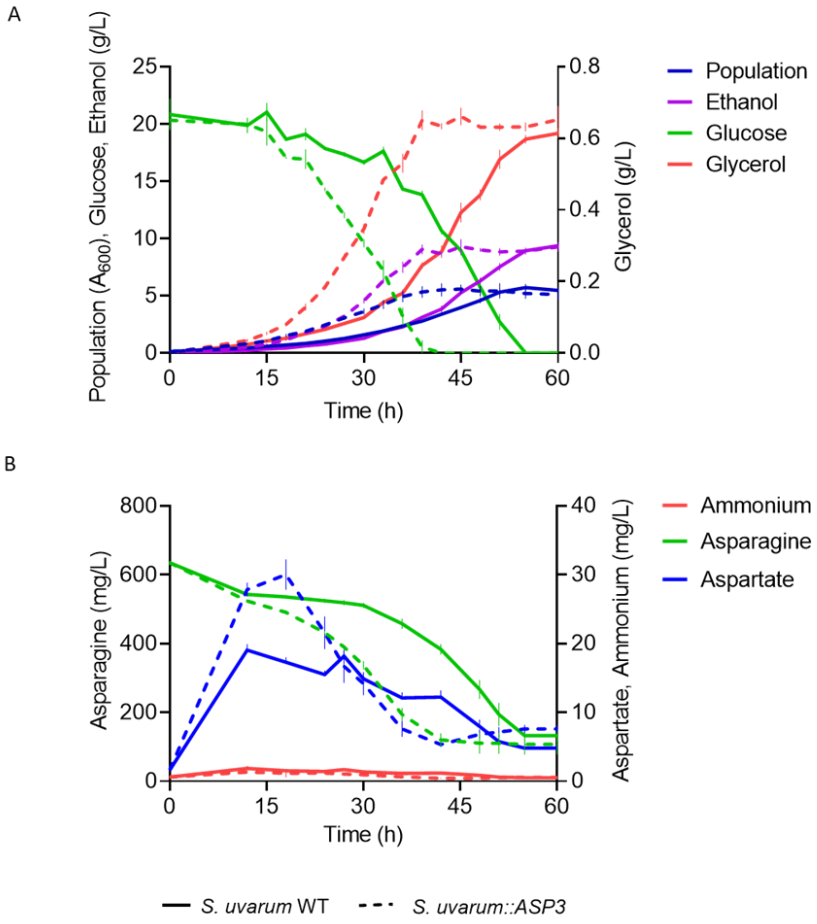

Figure 5. Evaluation of fermentation performance of *S. uvarum::ASP3*. *S. uvarum* wild type (*S. uvarum* WT) and the strain carrying *ASP3* (*S. uvarum::ASP3*) were grown in minimal medium containing glucose (20 g/L) and asparagine (100 mgN/L) at 30°C. (A) Fermentation parameters include population size expressed in A_{600}, glucose, ethanol and glycerol levels expressed in g/L. (B) Nitrogen consumption of asparagine, aspartate and ammonium are shown in mg/L. The continuous line represents *S. uvarum* WT and the dash line represents *S. uvarum::ASP3.* The error bars were calculated from biological triplicates.

Role of Asp3 in fermentation of apple juice

The growth advantage of *S. uvarum*::*ASP3* with asparagine as a nitrogen source led us to investigate how this strain would perform in apple juice,

where it is known that asparagine is present at higher concentrations than other amino acids. In the natural apple juice we used, the concentrations of asparagine, aspartate and NH_4 were 95 mgN/L, 10 mgN/L and 0.6 mgN/L, respectively. Despite the presence of asparagine as the main nitrogen source, however, the fermentation profiles of WT and *S. uvarum*::*ASP3* were indistinguishable (Figure 6A). Furthermore, the consumption patterns for the different nitrogen sources present were largely identical, with ammonium depleted first, then aspartate and finally asparagine (Figure 6B). There were also no detectable differences in the production of central carbon metabolites and the only variation at all was a transient reduction in α-ketoglutarate production in *S. uvarum*::*ASP3* (Supplementary Figure 5).

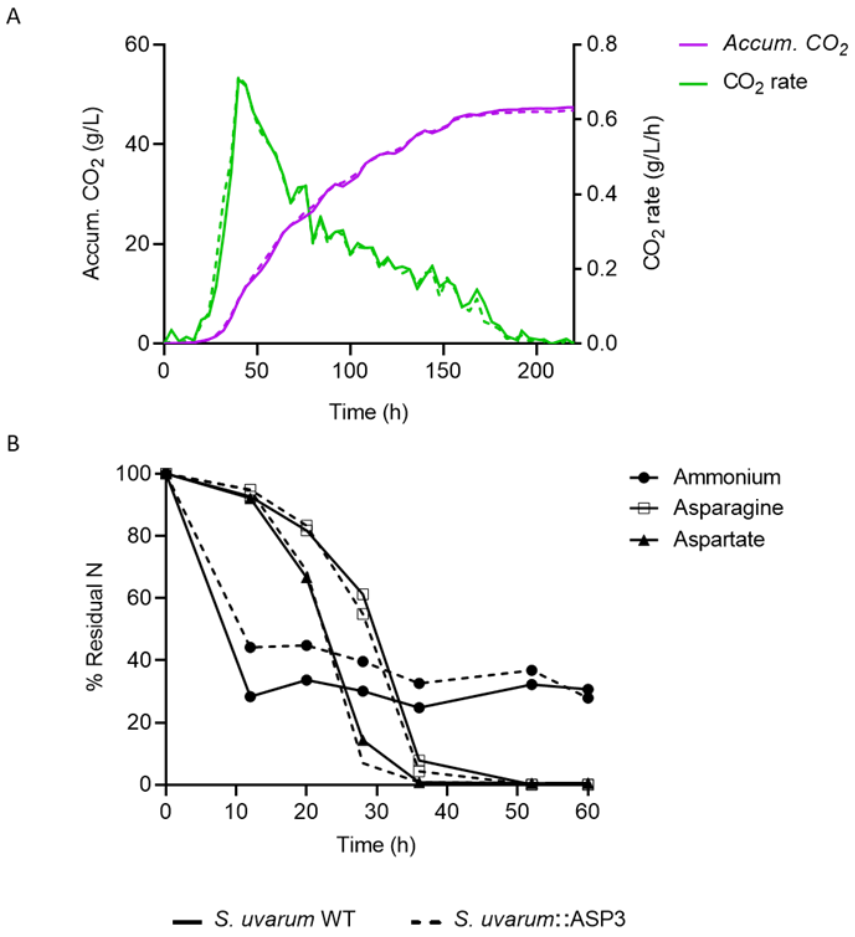

Figure 6. Fermentation profile in apple juice. Apple juice fermentations with *S. uvarum* wild type (WT) and *S. uvarum* carrying *ASP3* in the genome (*S. uvarum*::*ASP3*). (A) The progress of the fermentation is presented by showing the rate of CO_2 production (CO_2 rate) and the

total accumulated CO_2 (Accum. CO_2). The curves represent the mean of three replicates and were smoothed with 4 neighbouring points to average. (B) Nitrogen consumption during 60 hours of fermentation, shows the residual nitrogen through time expressed in percentage. The error bars were calculated from triplicates.

To understand why heterologous expression of *ASP3* conferred a growth advantage in MM but not in apple juice where asparagine is the main source of nitrogen, we performed a series of experiments varying different parameters, namely temperature, pH, nitrogen concentration and sugar content (Figure 7). These experiments compared wild-type and *S. uvarum*::*ASP3* using growth as the sole measurement of strain performance. The previous observation that *S. uvarum*::*ASP3* had a growth advantage in MM with asparagine as a sole nitrogen source was evident regardless of when asparagine was limiting (100 mgN/L) or in excess (1059 mgN/L), though the slower growth at 20^{o}C partially obscured the effect at that temperature. When a mixture of nitrogen compounds that mimic the nitrogen content of apple juice was used, the results were the same as with the apple juice. An effect of pH was excluded since raising pH of apple juice from pH 3.5 to pH 7 or reducing the pH of the MM to pH 3.5 had no impact. The final variable assessed was sugar concentration, so MM was prepared with sugar at 100 g/L as opposed to 20 g/L. The concentration of 100 g/L and the glucose:fructose ratio of 30:70 was designed to mimic that of apple juice. As before, in the nitrogen mix, both strains grew equally well and an advantage was seen for *S. uvarum*::*ASP3* when asparagine was limiting. It was interesting to note that of all the conditions tested, the advantage conferred by *ASP3* was greatest in this final condition, an indication perhaps that either the sugar concentration or the ratio of carbon to nitrogen might have some effect. The overall conclusion remains, however, that a benefit of carrying *ASP3* was only seen in circumstances where asparagine is the sole nitrogen source.

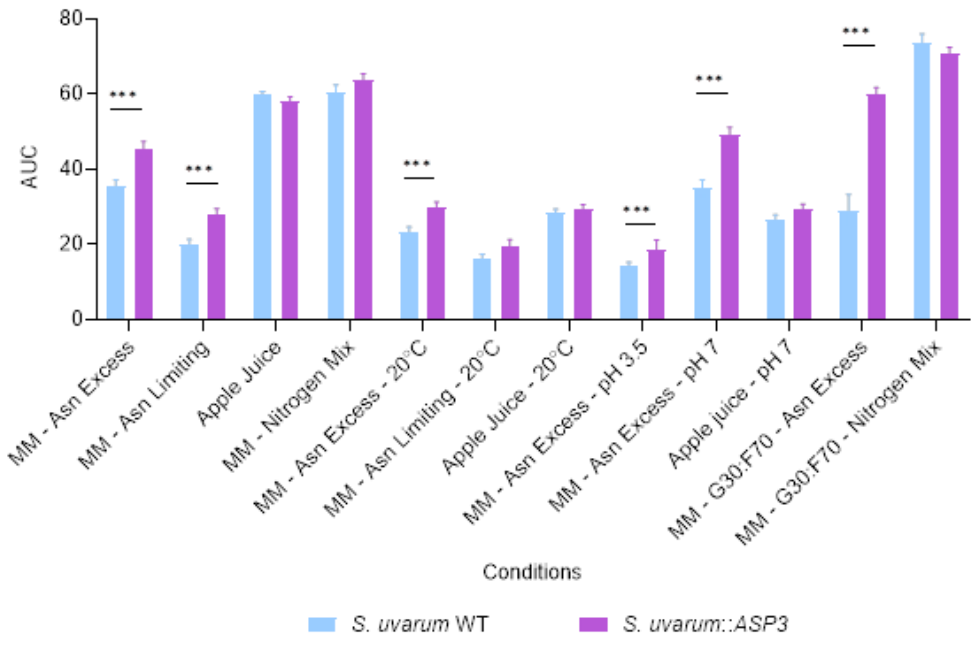

Figure 7. Evaluation of the growth of *S. uvarum* strains under different conditions. *S. uvarum* wild type (*S. uvarum* WT) and the *S. uvarum* strain carrying *ASP3* (*S. uvarum*::ASP3) were grown in different fermentation conditions for 60 hours and growth is presented using the "area under the curve (AUC)" parameter. The standard conditions were: Minimal medium (MM) containing 20 g/L glucose, excess concentration of nitrogen source (1059 mgN/L), pH of 4.6, at 30°C. When mentioned, just some factors varied from the standard conditions. Mix of nitrogen compounds (Nitrogen Mix) mimics the nitrogen composition of apple juice (127 mgN/L from which 95 mgN/L is asparagine). Limiting concentrations of asparagine represent 100 mgN/L (Asn Limiting). To variate the C:N ratio, 30 g/L of glucose and 70g/L of fructose (G30:F70) were used mimicking the sugar composition of apple juice. The pH was tested at 3.5 and 7. The error bars were calculated from five biological replicates. The *** show significant strain effect where p-value was <0.001.

DISCUSSION

There are many potential nitrogen sources available to fermenting yeast and cells have tight control mechanisms to enable efficient use of available resources. When provided with a mixture of inorganic and organic nitrogen, as is typical in a natural environment, these mechanisms allow cells sequentially use nitrogen sources in order of preference (Beltran, Novo, Rozes, Mas, & Guillamon, 2004; Crépin et al., 2012). In such beverage fermentations, nitrogen is generally limiting and the capacity to assimilate available nitrogen determines the rate of fermentation and the profile of AA-derived volatiles that are synthesised. The study of these processes is

therefore of interest from genetic, evolutionary, and biotechnological perspectives. Our focus was on the assimilation of asparagine and the evolution and role of the periplasmic asparaginase encoded by *ASP3*. Previous studies reported that this gene was only rarely found in *S. cerevisiae* and an attractive hypothesis was presented whereby *S. cerevisiae* acquired *ASP3* by HGT from *W. anomalus,* another yeast found in biotechnological environments (League et al., 2012). We tested this hypothesis bioinformatically and, in fact, the evidence was not supportive. Whilst we confirmed the low prevalence (~7%) of *ASP3* in *S. cerevisiae* by examining 1680 genome sequences, the distribution of *ASP3* in multiple clades of the *S. cerevisiae* phylogenetic tree and the syntenic location in all genomes where this could be determined, point to an ancestral origin with multiple losses being more likely than recent HGT in human associated fermentations. *ASP3* was not found in >20 genomes of the related species *S. uvarum* but the experience with *S. cerevisiae* indicates that many more genomes would be required to exclude the possibility that a percentage of strains encode Asp3. The detection of *ASP3* in the genomes of other budding yeasts could be taken as an indication that this gene is ancestral in this sub-phylum, but again more genome sequences are required before forming definitive conclusions.

Although we did not find evidence of HGT, with the exception of wine, there was enrichment in isolates from fermentative environments. This suggests that there was selection for *ASP3* positive strains, or for retention of *ASP3*, in strains used for brewing beer or making cider. The distinction between different beverage fermentations can be explained since asparagine is a prominent free amino acid in beer wort and apple juice, but not in grape must. Despite this, there is not any evidence that *ASP3* confers an advantage to *S. cerevisiae* since we found that strains with or without *ASP3* grew equally well when asparagine was the sole nitrogen source (Figure 3). It is possible, however, that the different genetic backgrounds of these strains occlude effects of Asp3. To more specifically address the hypothesis that

Asp3 would confer a growth advantage in apple juice, we expressed *ASP3* in a strain of *S. uvarum* that lacked an endogenous copy. Indeed, we found that growth was enhanced when asparagine was the sole nitrogen source but were surprised that this effect was not also observed in apple juice when asparagine is by far the most abundant source of nitrogen. Detailed analysis of the fermentation kinetics of the strain expressing *ASP3* on synthetic medium confirmed that Asp3 cleaved extracellular asparagine to ammonium and aspartate. As a preferred nitrogen source, the NH_4 was immediately assimilated via a Mep transporter and aspartate accumulated outside the cell to be assimilated later in the fermentation by either a specific or a general amino acid permease. It is not certain why the same benefit is not seen when a mixture of nitrogen sources is provided but we postulate that it relates to regulation via the NCR and SPS systems. Asparagine is not a preferred nitrogen source for *S. uvarum,* and we propose that when an alternative nitrogen source is present at low concentration, it is used first, allowing growth but also triggers the release from NCR as its availability drops. This increases expression of the general AA permeases *GAP1* and *DIP5*, and the asparagine-preferring permeases *AGP1* and *GNP1*, which allows the rapid uptake of asparagine and catabolism via Asp1 and thus no growth impairment is seen.

Other factors that should be considered when interpreting the data are species differences, genetic background of the strains, and additional selective pressures that have shaped strain domestication and evolution. The explanation for why differences are seen between when asparagine is the sole nitrogen source or is simply the dominant nitrogen source, highlights the importance that regulation can play. Genetic regulatory circuits are complex systems and already there are multiple examples of rewiring over the course of yeast evolution (Solieri et al., 2021). Thus, while components are conserved, it cannot be assumed that nitrogen regulatory systems function identically in *S. cerevisiae* and *S. uvarum*. Within *S. uvarum,* strain-specific variation is already evident as it was seen that some strains

are more effective assimilators of asparagine than others (Figure 3). Although the total numbers of strains tested was low (7), it was notable that two strains isolated from cider fermentations exhibited far better growth on asparagine than four strains from wine fermentations. This could indicate that, even without *ASP3*, cider strains have evolved to more efficiently use asparagine, the main source of nitrogen in apple juice, possibly by reprogramming regulatory circuits.

While our study provided some insights on Asp3 and its possible role in alcoholic fermentations, important questions remain. More genome sequences are needed to resolve whether *ASP3* truly is ancestral in budding yeasts, and, if so, why has it apparently been lost from so many species? This suggests that its original purpose is generally no longer relevant. Yet, even within *S. cerevisiae*, a minority of strains have retained the gene. The enrichment of *ASP3* positive strains in beer and cider fermentations would generally be taken to indicate an advantage for these strains but we did not see this. It should also be recognised, however, that historical selection of strains did not take place with pure cultures and interactions with other microbes could have been important. For example, in a competitive environment, *ASP3* strains may outcompete others for asparagine assimilation. Our data showing transient extracellular accumulation of aspartate was also interesting and it could be envisioned that another microbe would use this amino acid and provide a nutrient in return. Although we do not have evidence for this, similar symbiotic interactions are well-documented in fermented beverages (Comitini, Agarbati, Canonico, & Ciani, 2021; Stadie, Gulitz, Ehrmann, & Vogel, 2013; Tran et al., 2020). Indeed, it was shown that extracellular amino acids can play an important role in *Saccharomyces* community dynamics (Campbell, Herrera-Dominguez, Correia-Melo, Zelezniak, & Ralser, 2018; Mülleder et al., 2016). The possibility that Asp3 has alternative functions also cannot be excluded. Glutaminase activity for instance, has been reported to be a minor activity of asparaginases of yeast, and especially in bacteria and plants (Borek &

Jaskólski, 2001; Imada, Igarasi, Nakahama, & Isono, 1973; Michalska & Jaskolski, 2006; Sanches, Krauchenco, & Polikarpov, 2012), as well as the ability to transport dipeptides (Homann, Cai, Becker, & Lindquist, 2005). It is possible that some Asp3 variants improved one of these complementary activities in strains that retained the gene. It is clear that further investigations are required but, in overall terms, our study demonstrates how the integration of bioinformatic and experimental approaches can test evolutionary hypothesis and yield new insights of relevance for biotechnology.

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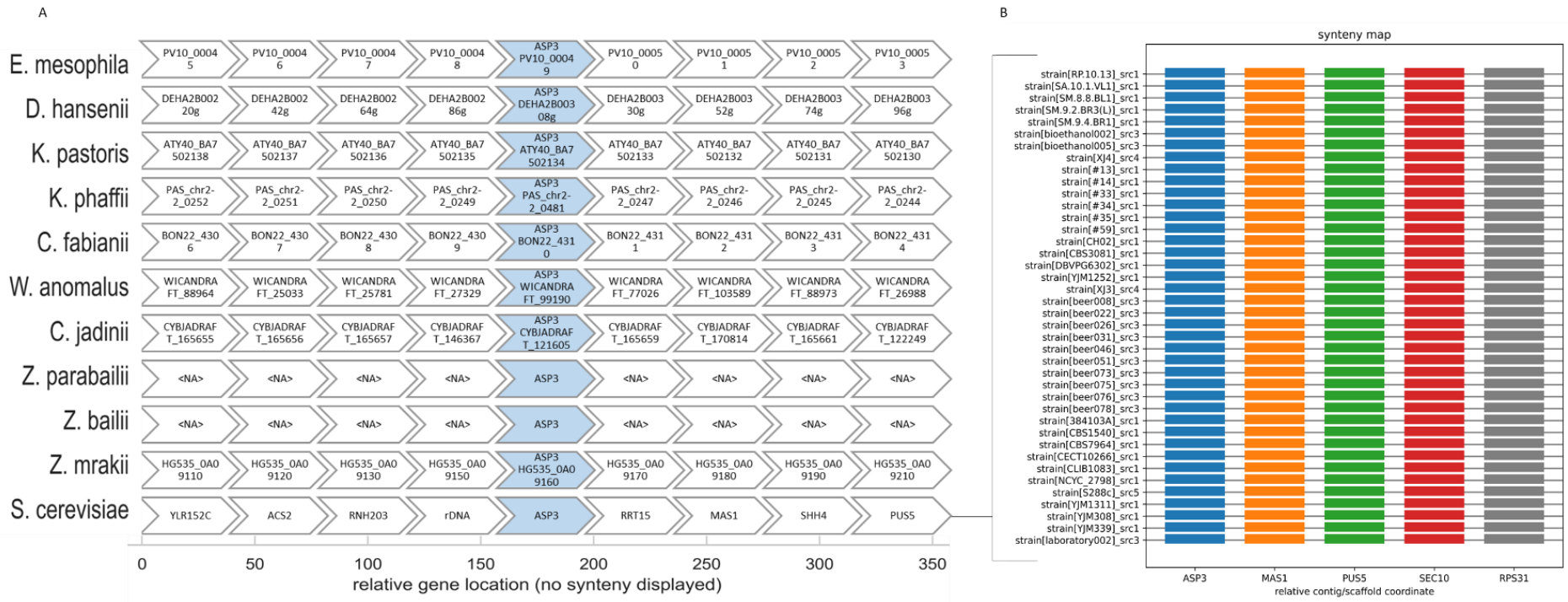
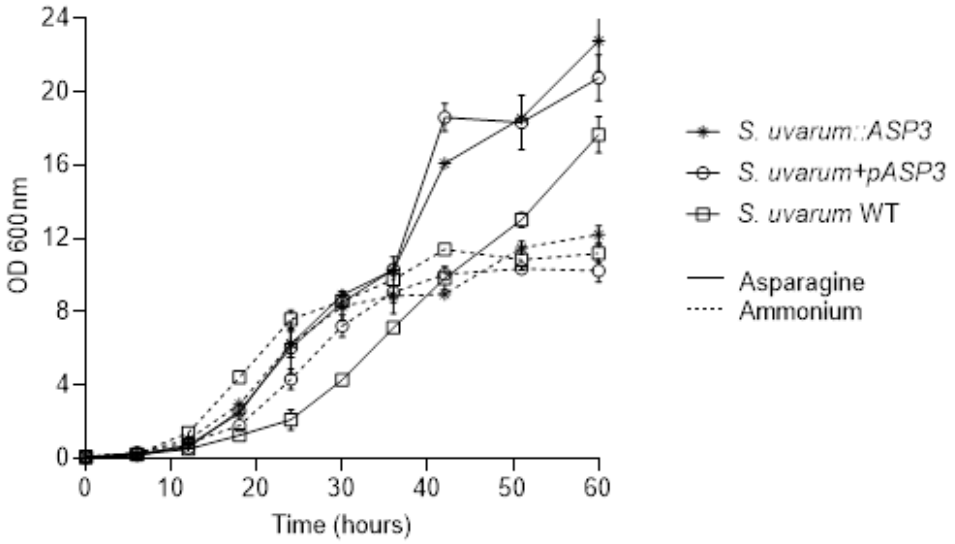
Conflict of Interest The authors do not report any conflict of interest.

SUPPLEMENTARY MATERIAL

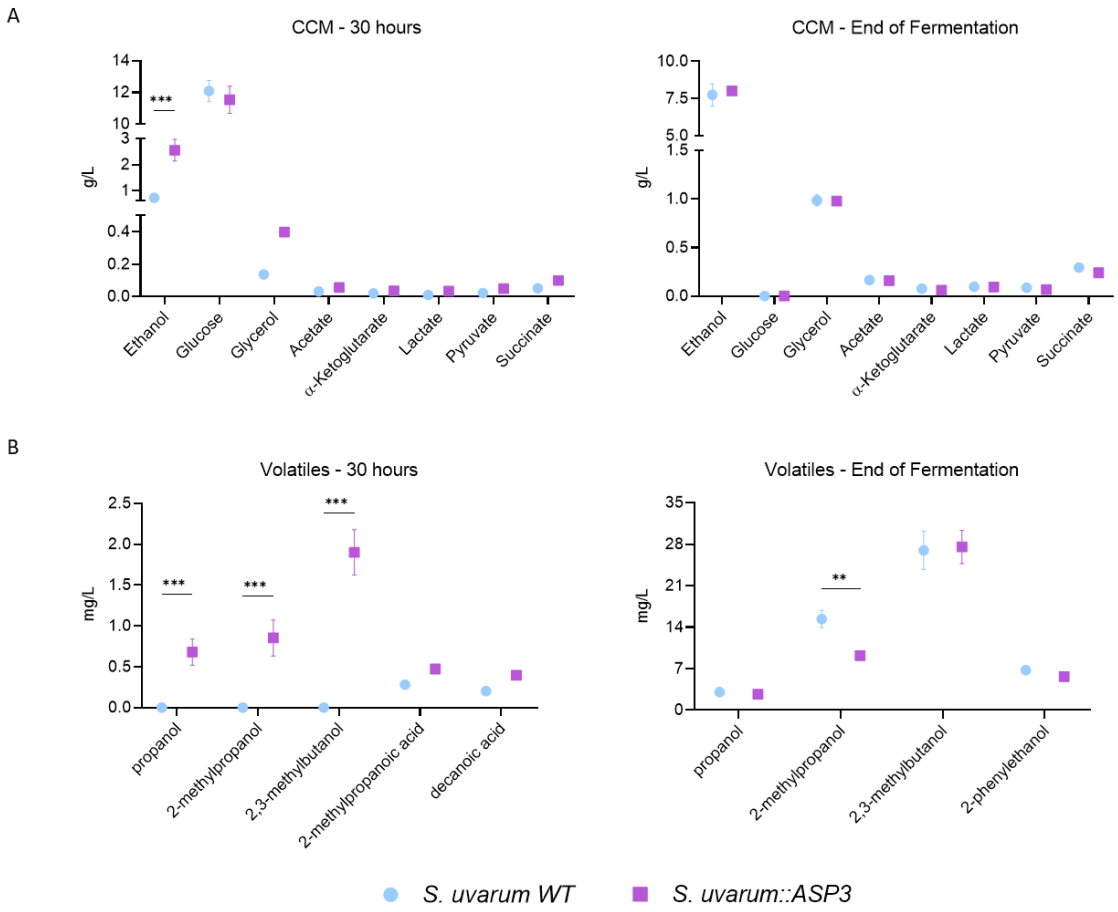
Supplementary Figure 1. Expanded phylogenetic tree of *ASP3* distribution in *S. cerevisiae* strains. Expanded version of Figure 2A.



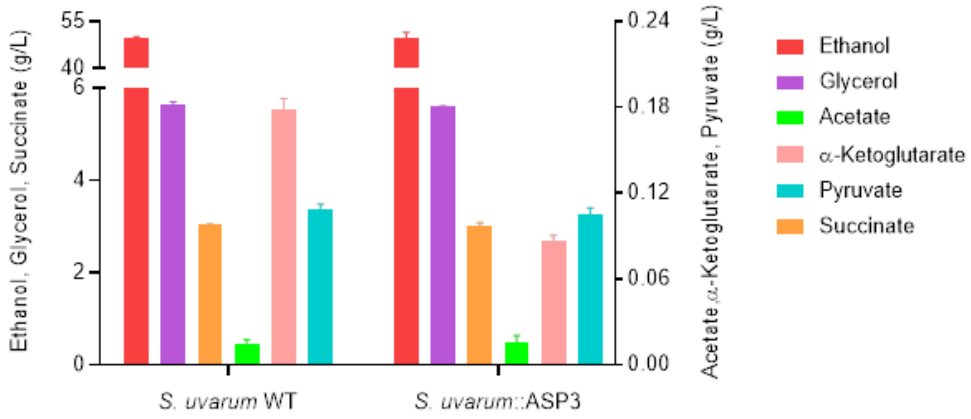
Supplementary Figure 2. Synteny analysis of *ASP3*. (A) Location of *ASP3* in 11 yeast species. *ASP3* and upstream/downstream genes in respective species. <NA> means that the gene is not annotated or that *ASP3* is on a contig by itself (the case of *Z. parabailii* and *Z. bailii*). (B) Synteny map of *ASP3-SEC10* locus *(ASP3-MAS1-PUS5-SEC10*) of 40 *S. cerevisiae* strains.



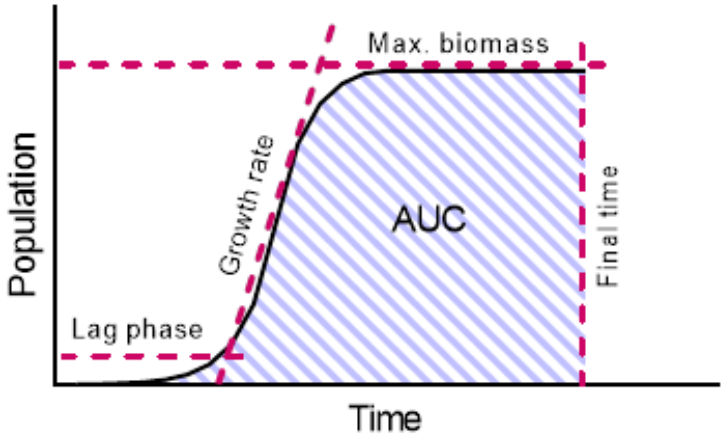
Supplementary Figure 3. *ASP3* improves growth of *S. uvarum* when asparagine is the sole nitrogen source. Growth curves of *S. uvarum* wild type (*S. uvarum* WT), *S. uvarum* complemented with *ASP3* plasmid (*S. uvarum*+pASP3) and *S. uvarum* with *ASP3* inserted in the genome (*S. uvarum*::*ASP3*). Strains grown on Minimal Medium with only asparagine as nitrogen source in excess (1059 mgN/L), at 20°C, for 60 hours. The curves represent the mean of four biological replicates.



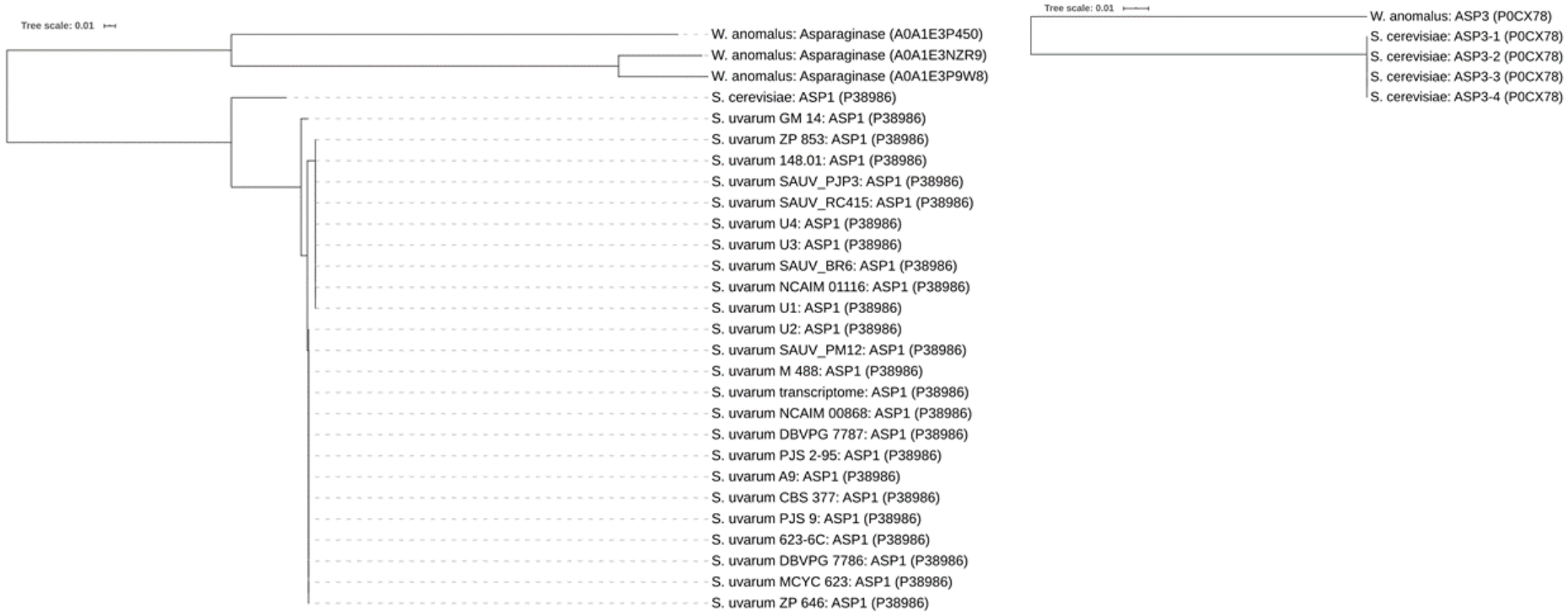
Supplementary Figure 4. Metabolites produced during fermentation. Differences on metabolites production between *S. uvarum* wild type (*S. uvarum* WT) and the strain carrying *ASP3* (*S. uvarum::ASP3)*. Fermentations done in minimal medium containing glucose 20 g/L and asparagine 1059 mgN/L at 20°C. Sampled at 0.3 fermentation progress point (30 hours) and at the end of fermentation (144 hours). (A) Central carbon metabolites (CCM) and glucose measured by HPLC, expressed in g/L. (B) Volatile compounds produced (6 compounds out of 34 analysed), expressed in mg/L. Statistical analysis done with Two-way ANOVA. The *** indicate significant difference between the strains.



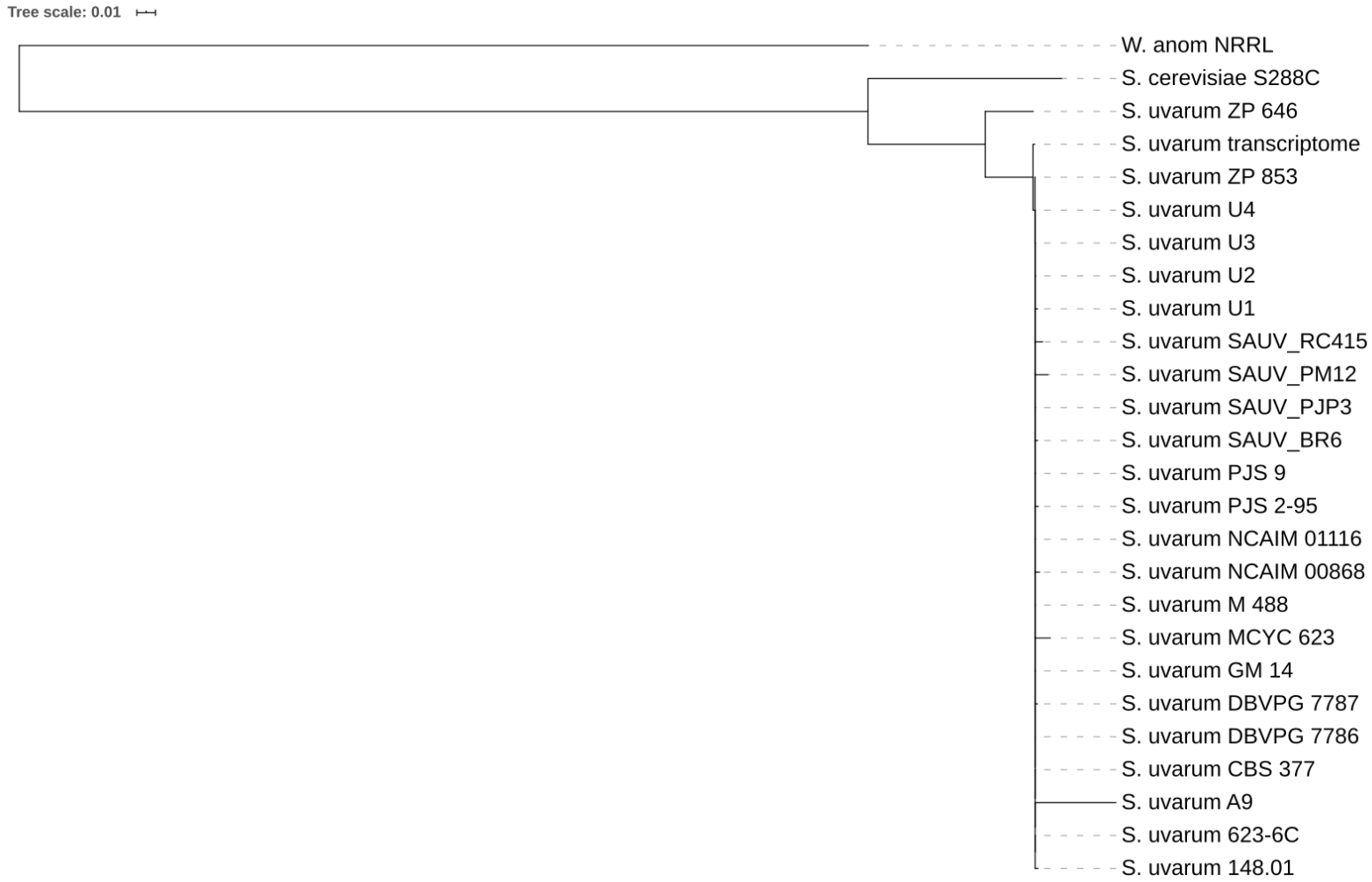
Supplementary Figure 5. Central carbon metabolites produced in apple juice fermentations. Apple juice fermentations performed with *S. uvarum* wild type (*S. uvarum* WT) and the *S. uvarum* strain carrying *ASP3* (*S. uvarum*::*ASP3*). Concentration of central carbon metabolites measured at the end of fermentation, expressed in g/L. The error bars were calculated from biological triplicates. Significant difference only found in α-ketoglutarate.



Supplementary Figure 6. Area under the curve (AUC) of population Vs time. AUC represent growth measurement, accounting for information of the lag phase length, growth rate, maximum biomass produced and final time.



Supplementary Figure 7. Phylogeny of the genes in the orthogroups containing ASP1 (left) and ASP3 (right). Species names, gene names and UniProt accession numbers are provided.



Supplementary Figure 8. Rooted tree of the species used in the proteome-wide homology matching using OrthoFinder.

Supplementary Table 1. Primers

Primer name	Primer sequence 5'-3'	Source
ASP3-cerevisiae-F	ATGAGATCTTTAAATACCCTTTTACTTTCT	This study
ASP3-cerevisiae-R	TTAACCACCGTAGACGCC	This study
ASP3-detection-F	TTTGGTACMGGYGGTACTAT	This study
ASP3-detection-R	AATAATBACTTCTGGAARTTC	This study
Seq-ASP3-cerevisiae-F	TTCTGAGAAATCACTGGGTCG	This study
Seq-ASP3-cerevisiae-R	TCATTTTCGTTGTCCAAAACC	This study
ASP1-uvarum-F	TATCTTTCTTTTTGCTTGGCTTTGC	This study
ASP1-uvarum-R	TGCACAATGACTTTTATGAAATCAAC	This study
Seq-ASP1-uvarum-F	GCCCATGAACCTTTACCAAG	This study
Seq-ASP1-uvarum-R	CGAAACAAGAACACTTCTTCC	This study
RF-HO-Uvarum-F	TCTTGCTTCCGTCGAAGTTCGTTAAAGTGTTCTAAATGTACTGTTAATCATCGAATCTGTCAGAACAGTA	This study
RF-HO-Uvarum-R	TTCTAATTTCATCATTTCATATAAAAGAAAATCCTGCAAATACTGTTCTGACAGATTCGATGATTAACAG	This study
DIAG-HO-F	ACTAGCTTCTGGGCGA	This study
DIAG-HO-R	TCACAATTAAAACTTTTTGTTTGCATTCA	This study
RF-ASP3insert-HO-F	TCTTGCTTCCGTCGAAGTTCGTTAAAGTGTTCTAAATGTACTGTTAATCATCGAAAGGAATAACGCATAGAGCC	This study
RF-ASP3insert-HO-R	TTCTAATTTCATCATTTCATATAAAAGAAAATCCTGCAAATACTGTTCTGACAGAACCTTTAGACTTACGTTTGCTAC	This study
ASP3-into-pgreg505-F	GAATTCGATATCAAGCTTATCGATACCGTCGATGAGATCTTTAAATACCCTTTTACTTTCT	This study
ASP3-into-pgreg505-R	GCGTGACATAACTAATTACATGACTCGAGGTCGACTTAACCACCGTAGACGCC	This study
RF-pASP3-GREGinsert-HR1a-F	GATATGATTTTGAAGTGTAACGCTAAGAAGAAAGAGGAACAAGCACAATACCACACACCATAGCTTCAAA	This study
RF-pASP3-GREGinsert-HR2a-R	CATTGTTTATATAATATGAAGCAAATTTAATAAAGAAAATAAATTTCAAGCGCAAATTAAAGCCTTCGAGCGT	This study
pGREG505-F	CCTCGAGTCATGTAATTAGTTATGTCACGC	Varela et al, 2019
pGREG505-R	CGACGGTATCGATAAGCTTGATATCGAA	Varela et al, 2019
BSA-clon-R	TACACGCGTTTGTACAGAAAAAAAAGAAAAATTTGA	

Supplementary Table 2. *Saccharomyces* strains used for *ASP3* detection by PCR.

Yeast	Strain	*ASP3*
Saccharomyces cerevisiae	S288c	Present
Saccharomyces cerevisiae	CEN.PK113-7D	Present
Saccharomyces cerevisiae	CEN.PK112	Present
Saccharomyces cerevisiae	EC1118	Absent
Saccharomyces cerevisiae	OS140	Absent
Saccharomyces cerevisiae	OS230	Absent
Saccharomyces cerevisiae	OS204	Absent
Saccharomyces cerevisiae	OS176	Absent
Saccharomyces cerevisiae	OS180	Absent
Saccharomyces cerevisiae	OS411	Absent
Saccharomyces uvarum	BMV58/MTF3098	Absent
Saccharomyces uvarum	CBS7001/OS24	Absent
Saccharomyces uvarum	OS472	Absent
Saccharomyces uvarum	CBS395	Absent
Saccharomyces uvarum	CBS7001	Absent
Saccharomyces mikatae	OS18	Absent
Saccharomyces kudriavzevii	OS130	Absent
Saccharomyces arboricola	OS350	Absent
Saccharomyces jurei	OS699	Absent
Saccharomyces paradoxus	OS6	Absent
Saccharomyces eubayanus	OS675	Absent

Supplementary Table 3. *S. cerevisiae* strains that carry *ASP3* clustered based on category. Same colour code as Figure 2 in manuscript.

Strain	Isolation source	Location	Data Source	Category
#33	Evolution canyon	Israel	1002 isolates - Peter et al. 2018	Nature
#34	Evolution canyon	Israel	1002 isolates - Peter et al. 2018	
#35	Evolution canyon	Israel	1002 isolates - Peter et al. 2018	
#13	Evolution canyon	Israel	1002 isolates - Peter et al. 2018	
#14	Evolution canyon	Israel	1002 isolates - Peter et al. 2018	
#59	Evolution canyon	Israel	1002 isolates - Peter et al. 2018	
#60	Evolution canyon	Israel	1002 isolates - Peter et al. 2018	
CBS7765	Salmo gairdneri (rainbow trout)	Goteborg, Sweden	1002 isolates - Peter et al. 2018	
CBS1540	Tibi grains	Switzerland	1002 isolates - Peter et al. 2018	
CCY_21-4-106	River water (Morava)	Devinska Nova Ves, Slovakia	1002 isolates - Peter et al. 2018	
2	Chalcidoidea	Schleswig-Holstein	1002 isolates - Peter et al. 2018	
3	Chalcidoidea	Forest of Nehmten Schleswig-Holstein	1002 isolates - Peter et al. 2018	
UCD_06-645	Female olive fly, surface sterilized	Davis, CA, USA	1002 isolates - Peter et al. 2018	
CLQCA_17-111	Termite mound	Ecuador	1002 isolates - Peter et al. 2018	
EM93_3	Rotting fig - Fruit	California, USA	1002 isolates - Peter et al. 2018	
CBS6333	Rotting banana (from Musa sapientum)	Costa Rica	1002 isolates - Peter et al. 2018	
SJ5L14	Fruiting body of Auricularia auricular	Taian, Miaoli, Taiwan	1002 isolates - Peter et al. 2018	
YJM1383	Coconut	Philippines	1002 isolates - Peter et al. 2018	
CLIB1070	Dry cider	Lower Normandy, France	1002 isolates - Peter et al. 2018	
CLIB1071	Dry cider	Lower Normandy, France	1002 isolates - Peter et al. 2018	
CLIB1072	Dry cider	Lower Normandy, France	1002 isolates - Peter et al. 2018	
CLIB1077	Dry cider	Upper Normandy, France	1002 isolates - Peter et al. 2018	
CLIB1082	Dry cider	Upper Normandy, France	1002 isolates - Peter et al. 2018	
CLIB1083	Dry cider	Upper Normandy, France	1002 isolates - Peter et al. 2018	
SRC 120	Cider bioindustrial strain	France	1002 isolates - Peter et al. 2018	

XJ3	Apple - Orchard	Korla, Xinjiang	Asian strain paper - Duan et al. 2018	Brewing & Cider
CBS1171	Brewers top yeast - Oranjeboom brewery	Rotterdam, Netherlands	1002 isolates - Peter et al. 2018	
CBS7371	Brewery	United Kingdom	1002 isolates - Peter et al. 2018	
CBS382	Beer	Rio de Janeiro, Brazil	1002 isolates - Peter et al. 2018	
CLIB227	Beer holland	The Netherlands	1002 isolates - Peter et al. 2018	
CH02	Pearl millet beer	Abengourou, Ivory Coast	1002 isolates - Peter et al. 2018	
CH06	Pearl millet beer	Abengourou, Ivory Coast	1002 isolates - Peter et al. 2018	
CH10	Pearl millet beer	Abengourou, Ivory Coast	1002 isolates - Peter et al. 2018	
CH14	Pearl millet beer	Abengourou, Ivory Coast	1002 isolates - Peter et al. 2018	
CH13	Pearl millet beer	Abengourou, Ivory Coast	1002 isolates - Peter et al. 2018	
DBVPG6694	Artois Peterman beer	Belgium	1002 isolates - Peter et al. 2018	
995	Fermented beverage from raisins	Hungary	1002 isolates - Peter et al. 2018	
beer008	Beer (Ale)	Germany	Beer strain paper - Gallone et al. 2016	
beer016	Beer	Belgium	Beer strain paper - Gallone et al. 2016	
beer022	Beer (Ale refermentation)	Belgium	Beer strain paper - Gallone et al. 2016	
beer026	Beer (Ale refermentation)	Belgium	Beer strain paper - Gallone et al. 2016	
beer031	Beer (Trappist)	Belgium	Beer strain paper - Gallone et al. 2016	
beer046	Beer	Germany	Beer strain paper - Gallone et al. 2016	
beer051	Beer	East Coast, USA	Beer strain paper - Gallone et al. 2016	
beer073	Beer (Hefeweizen)	Germany	Beer strain paper - Gallone et al. 2016	
beer075	Beer (Wheat)	Belgium	Beer strain paper - Gallone et al. 2016	
beer076	Beer (Wheat)	Belgium	Beer strain paper - Gallone et al. 2016	
beer078	Beer (Trappist)	Belgium	Beer strain paper - Gallone et al. 2016	
YJM436_1b	Mouth - Human	NAN	1002 isolates - Peter et al. 2018	Clinical/Human isolates
CBS2909	Human feces	NAN	1002 isolates - Peter et al. 2018	
CBS2910	Human feces	Portugal	1002 isolates - Peter et al. 2018	
YJM308	Human	NAN	1002 isolates - Peter et al. 2018	

YJM339	Human	NAN	1002 isolates - Peter et al. 2018	
384103A	Human - RVI	Newcastle, UK	1002 isolates - Peter et al. 2018	
2720	Blood - Human	Valencia, Spain	1002 isolates - Peter et al. 2018	
NCYC_2798	Clinical isolate, AIDS patient	Germany	1002 isolates - Peter et al. 2018	
SC 32 F. Dromer IP	Human feces	France	1002 isolates - Peter et al. 2018	
SC 33 F. Dromer IP	Human feces	France	1002 isolates - Peter et al. 2018	
Ponton 12	Mouth - Human	Spain	1002 isolates - Peter et al. 2018	
malade3285/11-2-4	Bronchoalveolar lavage - Human	Paris, France	1002 isolates - Peter et al. 2018	
malade 98 2601	Human feces	Amiens, France	1002 isolates - Peter et al. 2018	
378604X	Sputum - Human	Newcastle, UK	1002 isolates - Peter et al. 2018	
YJM248	Human feces	NAN	1002 isolates - Peter et al. 2018	
YJM451	Human	NAN	1002 isolates - Peter et al. 2018	
YJM693	Human	Texas, USA	1002 isolates - Peter et al. 2018	
YJM996	Human	Italy	1002 isolates - Peter et al. 2018	
YJM1311	Human	Michigan, USA	1002 isolates - Peter et al. 2018	
YJM1078	Human	Portugal	1002 isolates - Peter et al. 2018	
T73	Wine	NAN	Darren_SGD_strains	Wine
Y9J_1b	Wine	Japan	1002 isolates - Peter et al. 2018	
wine016	Wine	Italy	Beer strain paper - Gallone et al. 2016	
XJ4	Grape - Orchard	Korla, Xinjiang	Asian strain paper - Duan et al. 2018	
S288c	Laboratory	NAN	Darren_SGD_strains	Laboratory
BY4741	Laboratory	NAN	Darren_SGD_strains	
BY4742	Laboratory	NAN	Darren_SGD_strains	
W303	Laboratory	NAN	Darren_SGD_strains	
FL100	Laboratory	NAN	Darren_SGD_strains	
W303	Laboratory	NAN	1002 isolates - Peter et al. 2018	
S288C	Laboratory	NAN	AWRI genomics - Borneman et al. 2016	
laboratory002	Laboratory	NAN	Beer strain paper - Gallone et al. 2016	

CBS7964	Fermenting concentrated syrup (sugar cane)	Brazil	1002 isolates - Peter et al. 2018	Bioethanol & Food Industry
CBS7960	Factory producing ethanol (sugar cane syrup)	Sao Paulo, Brazil	1002 isolates - Peter et al. 2018	
CBS7959	Factory producing ethanol (sugar cane syrup)	Sao Paulo, Brazil	1002 isolates - Peter et al. 2018	
CBS7961	Fermenting concentrated syrup (sugar cane)	Sao Paulo, Brazil	1002 isolates - Peter et al. 2018	
CECT10266	Tanning liquor	Spain	1002 isolates - Peter et al. 2018	
RP.10.13	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SM.9.1.AL1	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SM.9.1.BL7	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SM.9.2.BR3(L)	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SM.9.4.BL2	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SM.9.4.BR1	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SM.8.2.C13	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SM.8.7.L8	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SM.8.7.L9	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SM.8.8.BL1	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SA.9.2.BL3	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SA.9.4.VL4	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SA.10.1.VL1	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
SA.10.1.VR4	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
RP11.4.1	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
RP11.4.11	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
RP11.4.14	Bioethanol	Brazil	1002 isolates - Peter et al. 2018	
bioethanol002	Bioethanol	Brazil	Beer strain paper - Gallone et al. 2016	
bioethanol005	Bioethanol	Brazil	Beer strain paper - Gallone et al. 2016	
CLIB324	Baking	Vietnam	Darren_SGD_strains	
TL229S2.2	Cheese - Dairy	France	1002 isolates - Peter et al. 2018	

CBS3081	Alpechin - Waste from olive oil production	Spain	1002 isolates - Peter et al. 2018	
DBVPG6302	Alpechin - Waste from olive oil production	Spain	1002 isolates - Peter et al. 2018	
UCD_09-448	Olives from Middle of season 1	Davis, CA, USA	1002 isolates - Peter et al. 2018	
UCD_11-601	Olive fermentation - Sicilian style olives - Pilot plant	Davis, CA, USA	1002 isolates - Peter et al. 2018	
1560	Manzanilla - Alorena, olive (Noe)	Aceituna, Spain	1002 isolates - Peter et al. 2018	
YJM1252	Alpechin - waste from olive oil production	Spain	1002 isolates - Peter et al. 2018	
CBS428a	Unknown	NAN	1002 isolates - Peter et al. 2018	Unknown
CBS3078	Spores - Unknown	NAN	1002 isolates - Peter et al. 2018	
AWRI934	Unknown	NAN	AWRI genomics paper - Borneman et al. 2016	
AWRI1729	Unknown	NAN	AWRI genomics paper - Borneman et al. 2016	

Supplementary Table 4. Accession information for *Saccharomyces uvarum* genome assemblies used.

Bioproject	GenBank ID	Assembly ID	Strain	Assembly level
PRJNA388544	GCA_002242675.1	ASM224267v1	U1	Scaffold
PRJNA388544	GCA_002242715.1	ASM224271v1	U2	Scaffold
PRJNA388544	GCA_002242645.1	ASM224264v1	U3	Scaffold
PRJNA388544	GCA_002242635.1	ASM224263v1	U4	Scaffold
PRJNA522928	GCA_013265745.1	ASM1326574v1	148.01	Scaffold
PRJNA522928	GCA_000167035.1	ASM16703v1	623-6C	Scaffold
PRJNA522928	GCA_000732305.1	ASM73230v1	A9	Contig
PRJNA522928	GCA_013265775.1	ASM1326577v1	CBS 377	Scaffold
PRJNA522928	GCA_013180345.1	ASM1318034v1	DBVPG 7786	Scaffold
PRJNA522928	GCA_013180255.1	ASM1318025v1	DBVPG 7787	Scaffold
PRJNA522928	GCA_013180235.1	ASM1318023v1	GM 14	Scaffold
PRJNA522928	GCA_013180195.1	ASM1318019v1	M 488	Scaffold
PRJNA522928	GCA_000166995.1	ASM16699v1	MCYC 623	Contig
PRJNA522928	GCA_013265705.1	ASM1326570v1	NCAIM 00868	Scaffold
PRJNA522928	GCA_013180055.1	ASM1318005v1	NCAIM 01116	Scaffold
PRJNA522928	GCA_013179965.1	ASM1317996v1	PJS 2-95	Scaffold
PRJNA522928	GCA_013179955.1	ASM1317995v1	PJS 9	Scaffold
PRJNA522928	GCA_013183795.1	ASM1318379v1	S6U	Scaffold
PRJNA522928	GCA_013181555.1	ASM1318155v1	SAUV_BR6	Scaffold
PRJNA522928	GCA_013180005.1	ASM1318000v1	SAUV_PJP3	Scaffold
PRJNA522928	GCA_013179935.1	ASM1317993v1	SAUV_PM12	Scaffold
PRJNA522928	GCA_013179855.1	ASM1317985v1	SAUV_RC415	Scaffold
PRJNA522928	GCA_013179785.1	ASM1317978v1	ZP 646	Scaffold
PRJNA522928	GCA_013179815.1	ASM1317981v1	ZP 853	Scaffold

Supplementary method. Investigating *ASP3* homology in *S. uvarum*

Methods

The homology of *ASP3* in *Saccharomyces uvarum* was investigated as follows. Genome assemblies for *Saccharomyces cerevisiae* S288C (RefSeq accession: GCF_000146045.2), *Wickerhamomyces anomalus* NRRL Y-366-8 (GenBank accession: GCA_001661255.1), and *Saccharomyces uvarum* assemblies from BioProjects PRJNA388544 & PRJNA522928 (Supplementary Table 4) were downloaded from NCBI Assembly. A *Saccharomyces uvarum* transcriptome was assembled from paired end RNA-seq reads of cultures grown in methionine, ammonium, asparagine and phenylalanine using Trinity (Grabherr et al., 2011). Genes in these assemblies were predicted using Augustus (version 3.3.3; Stanke et al. 2008), and the "getAnnoFasta.pl" script was used to extract the FASTA amino acid sequences of these predicted genes. The amino acid sequences for these predicted genes were used to perform proteome-wide homology matching using OrthoFinder (version 2.2.7; Emms and Kelly 2019). FASTA amino acid sequences for *ASP3-3* (accession: P0CX78) and *ASP1* (accession: P38986) were downloaded from UniProtKB/Swiss-Prot (The UniProt Consortium, 2019), and used to create a local protein BLAST database. The amino acid sequences of the predicted genes were searched against this database using BLASTp through BLAST+ (version 2.9.0; Camacho et al. 2009). Predicted genes were also searched against UniProtKB using BLASTp.

Results

Using BLASTp, *ASP3* could be identified in the *S. cerevisiae* and *W. anomalus* proteomes, but not in the *S. uvarum* proteomes. Of the 146,355 predicted genes used in the proteome-wide homology analysis, 142,383 (97.3%) were assigned to orthogroups. All the *ASP3* and *ASP1* proteins identified were present in one of two orthogroups (Supplementary Figure 7). *ASP3* was not identified in the *S. uvarum* proteomes (Supplementary Figure 8).

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Chapter 4

Regulated use of alternative Transcription Start Sites controls the production of cytosolic or mitochondrial forms of branched-chain aminotransferase in *Kluyveromyces marxianus*

Angela Coral-Medina[1,2], Darren A. Fenton[1,3], Javier Varela[1,4], Carole Camarasa[2], Pavel V. Baranov[3], John P. Morrissey[1]

[1]School of Microbiology, University College Cork, Ireland; [2]SPO, Univ Montpellier, INRAE, Institut Agro, Montpellier, France; [3]School of Biochemistry and Cell Biology, University College Cork, Ireland [4]Current address: Metabolic engineering department, CarboCode GmbH, Konstanz, Germany.

*Corresponding author: j.morrissey@ucc.ie

ABSTRACT

Following a whole genome duplication (WGD) event approximately 100 million years ago, the yeast lineage from which the model *Saccharomyces cerevisiae* derives maintained two copies of genes where it was necessary to synthesise proteoforms with different sub-cellular localisation. In contrast, yeasts that did not undergo the WGD event have a single gene that must encode both proteoforms. We adopted an integrated *in silico* and experimental approach to study how this is achieved with *BAT1*, a gene that encodes mitochondrial and cytosolic forms of a branched chain aminotransferase (BCAT) in pre-WGD yeast such as *Kluyveromyces marxianus*. We determined that condition-specific regulation of alternative transcription sites gives rise to mRNA isoforms that differ at the 5'end and that, when decoded, generate a mitochondrial or cytosolic proteoform. Furthermore, targeted mutants lacking specific transcription factors were generated to establish how this differentiation was regulated. As in *S. cerevisiae*, Gcn4 and Leu3 activated expression of the mRNA encoding the mitochondrial proteoform under conditions when branched chain amino acid synthesis was required. Unlike *S. cerevisiae*, however, *K. marxianus* lacked tight regulation of the mRNA encoding the cytosolic proteoform supporting the hypothesis that maintaining paralogous genes in post-WGD yeasts facilitated development of more sophisticated expression control mechanisms.

INTRODUCTION

Many volatile molecules derived from yeast metabolism have flavour and aroma properties that make significant contributions to fermented beverages and are of increasing interest for the food and cosmetic sectors (Dzialo, Park, Steensels, Lievens, & Verstrepen, 2017). Some of the most significant aromas and flavours are derived from the degradation (catabolism) of amino acids in the Ehrlich pathway to produce a range of

higher alcohols, acids and esters. These include the aromatic amino acids, phenylalanine and tyrosine, and the branched chain amino acids (BCAA), valine, isoleucine and leucine, and individual amino acids such as threonine and methionine (Hazelwood et al, 2008; Isogai et al., 2022). In each case, the first catabolic step is a transamination to form an α-ketoacid by distinct aromatic amino acid (AAT) or branched chain amino acid aminotransferases (BCAT). The reaction is reversible and, depending on the nutritional requirements of the cell, the pathway can be used for either the degradation or the biosynthesis of amino acids. There is a degree of pathway compartmentalisation with the aminotransferase catabolic reaction taking place in the cytosol and the reverse biosynthetic reaction in the mitochondrion. Although most work on flavour/aroma metabolism has been performed in the baker's yeast, *Saccharomyces cerevisiae*, the potential for non-traditional yeasts as sources of these metabolites has led to new interest in the study of amino acid metabolism and production of flavours and aromas in non-*Saccharomyces* species (Morrissey, Etschmann, Schrader, & de Billerbeck, 2015).

S. cerevisiae often deals with the challenge of producing cytosolic and mitochondrial variants of the same enzyme (proteoforms) by possessing two copies of the relevant gene, one of which encodes a protein with a mitochondrial targeting signal (MTS). Examples of this include *BAT1* and *BAT2,* or *AAT1* and *AAT2,* encoding BCAA and aspartate aminotransferases, respectively. These are paralogous genes (Kellis, Birren, & Lander, 2004) that arose from the whole genome duplication event that occurred approximately 100 million years ago when ancestral yeasts from the *Kluyveromyces*, *Lachancea* and *Eremothecium* (KLE) and *Zygosaccharomyces / Torulaspora* (ZT) clades hybridised to form a new lineage (Conant & Wolf, 2008; Marcet-Houben & Gabaldón, 2015; Wolfe, 2015). The presence of paralogous genes post-WGD enabled functional differentiation in some cases, for example, differential gene expression, alteration of kinetic properties, oligomeric organization or sub-cellular localisation of expressed

proteins (González et al., 2017; Quezada et al., 2008). In contrast, so-called "pre-WGD" yeast such as those in the KLE clade, retain the ancestral state of a single gene that must be responsible for both functions of the orthologous *S. cerevisiae* genes. Establishing how this can be achieved is important for understanding the metabolism and physiology of non-*Saccharomyces* yeasts. It also contributes to our understanding of some of the evolutionary processes that took place post-WGD leading to the specialisation and niche adaptation of *S. cerevisiae*.

BAT1, encoding the mitochondrial BCAT, and *BAT2,* the cytosolic one, are among the best studied aminotransferases in *S. cerevisiae*. Bat1 and Bat2 have 77% amino acid identity and can each perform the forward or reverse reaction but the Bat1 proteoform is localised in the mitochondrial matrix due to the presence of a mitochondrial targeting signal (MTS) at the amino terminus (Kispal et al., 1996). The different sub-cellular localisation allows functional specialisation of Bat1 for BCAA synthesis in the mitochondrion and Bat2 for BCAA degradation in the cytosol (Colon et al. 2011). *BAT1* and *BAT2* show different patterns of expression depending on the environmental nitrogen conditions and the related physiological requirements of the cell (González et al., 2017). The nitrogen-source control of *BAT1/BAT2* expression is tight, with both genes being subject to the activity of transcriptional regulators. In *S. cerevisiae* grown in biosynthetic conditions (for example using NH_4 or glutamine as a sole nitrogen source), the transcription factors (TFs) Gcn4 and Leu3 directly bind to the *BAT1* promoter activating expression. The activating function of Leu3 requires that it is bound by α-isopropylmalate (α-iPM), an intermediate of the leucine biosynthetic pathway – in the absence of (α-iPM), Leu3 represses transcription. There is no *BAT2* expression under these conditions and nucleosomes occlude much of the promoter. In contrast, under catabolic conditions (BCAA as a nitrogen source), *BAT1* is repressed and *BAT2* is activated. The activation of *BAT2* involves the nitrogen catabolite repression (NCR) activator Gln3, which translocates to the nucleus under these

conditions and directly binds the *BAT2* promoter, driving expression. A second TF, Put3, also binds the *BAT2* promoter and has an activating effect. Put3 plays a second role in this system as it represses *LEU1*, which is required for α-iPM synthesis. The effect of this is that the Leu3 bound to the *BAT1* promoter is not bound by α-iPM and so represses *BAT1* expression. As these are amino acid-replete conditions, Gcn4 levels are low so this activator is absent and thus cannot overcome the repressive effect of Leu3.

Previous studies of the BCAT genes in pre-WGD yeasts, *Kluyveromyces lactis* and *Lachancea kluyveri* reported that two proteoforms with opposing functions are encoded by a single gene *BAT1* (Colón et al., 2011; Montalvo-Arredondo et al., 2015). Those studies proposed that the biosynthetic and catabolic roles of the ancestral Bat1 have been partitioned in *BAT1* and *BAT2* through differential expression and subcellular re-localisation, improving regulation of BCAA metabolism in *S. cerevisiae* (Montalvo-Arredondo et al., 2015). A number of questions remained unresolved from the previous studies. First, how does the single *BAT1* gene give rise to both cytosolic and mitochondrial proteoforms? Second, how is that process regulated and controlled? Addressing this leads to a further question as to whether the nitrogen source-dependent transcriptional regulation of *BAT1* and *BAT2* that is seen in *S. cerevisiae* is derived from ancestral regulation or was newly-acquired in post-WGD yeasts? We chose to address these questions in *Kluyveromyces marxianus* for three main reasons. First, a recently published multi-omics based annotation of *K. marxianus* provides a level of genome-wide resolution on gene structure and expression and gives the raw data to investigate the *BAT1* locus in detail (Fenton, Kiniry, et al., 2022; Fenton, Świrski, et al., 2022). Second, the CRISPR-based genome engineering tools developed for *K. marxianus* enable rapid and precise genome editing thereby facilitating strain engineering to address hypotheses that are generated (Rajkumar & Morrissey, 2022). And finally, because *K. marxianus* is emerging as one of the most important new yeasts for biotechnology applications, there is an imperative to improve our knowledge of its genetics

and physiology (Fonseca, Heinzle, Wittmann, & Gombert, 2008; Homayouni-Rad, Azizi, Oroojzadeh, & Pourjafar, 2020; Karim, Gerliani, & Aïder, 2020; Leonel, Arruda, Chandel, Felipe, & Sene, 2021; Morrissey et al., 2015).

This study set out with the aim of understanding how *K. marxianus* manages to produce each Bat1 proteoform depending on the metabolic needs. Initially, the structure of the *BAT1* locus was examined in the GWIPS-Viz genome browser (Michel, Kiniry, O'connor, Mullan, & Baranov, 2017) to identify features such as transcriptional start sites (TSS) (TSS), mRNA translation and ORF architecture to examine potential alternative translation start sites. Next, using protein fusions, we demonstrated the presence of an MTS at the N-terminus of Bat1. Expression analysis confirmed that *BAT1* uses alternative TSS to produce the two differentially localised Bat1 proteoforms, with the choice of TSS determined by the nitrogen status of the cells. To investigate this further, we individually inactivated eight transcript factors (TFs) and found that several of those involved in regulation of *BAT1* and *BAT2* in *S. cerevisiae* also play a role in *BAT1* regulation in *K. marxianus*. The main similarity was the positive effect of Gcn4 and Leu3 on expression of the mitochondrial form of BCAT. There was a divergence with the cytosolic form, however, with the data indicating the paralogue *BAT2* acquired positive regulation via Gln3 and Put3 following the whole genome duplication.

MATERIALS AND METHODS

Strains and culture conditions

All *K. marxianus* strains used in this study are derived from *K. marxianus* NBRC1777 and are listed in Table 1. Strains were routinely cultivated in YPD broth (2% glucose, 1% yeast extract, 2% peptone) at 28°C. For experiments with specific nitrogen sources, a minimal media (MM) based on the standard Verduyn medium was used (glucose 20 gL^{-1}, KH_2PO_4 3 gL^{-1}, $MgSO_4.7H_2O$ 0.5 gL^{-1}, vitamin mix, and trace elements mix) (Verduyn et al., 1992). This was

supplemented with individual nitrogen sources as follows: ammonium sulfate 5 gL^{-1}, glutamine 5.5 gL^{-1}, valine 8.8 gL^{-1}, isoleucine 9.9 gL^{-1}, leucine 9.9 gL^{-1}, or a mixture of BCAAs termed VIL (valine 150 mgL-1, isoleucine 30 mgL-1, leucine 100 mgL-1). For tests on different N-sources, 10 mL cultures were pre-grown on YPD for 16h, harvested by centrifugation, and resuspended in Yeast Nitrogen Base (YNB) (glucose 20 gL^{-1}) media without amino acids and ammonium sulphate. Incubation was continued for further 4h to deplete cellular nitrogen reserves and the cells were harvested again, washed with 0.9% NaCl saline solution, before resuspension in the desired growth medium at an initial A_{600} of ~0.05. Subsequent growth tests were performed either in flasks or microtitre plates. When required for selection and plasmid maintenance, G418 was used at 200 ng/µL.

Batch growth was performed in 100 mL flasks containing 25 mL of MM supplemented with the appropriate nitrogen source. The cultures were inoculated at initial A_{600} ~0.05. Experiments were performed in triplicate. For the microplate fermentations the A_{600} of the starter cultures was set to 0.5 to then inoculate fresh media at ~0.05 initial A_{600}; the total volume in each well was 200 µL. This experiment was done with a minimum of four biological replicates of each strain in each media (ammonium sulfate, VIL, glutamine, valine, isoleucine, leucine, ammonium+VIL), using one blank per media. The 96 flat wells microplate was incubated in the microplate reader CLARIOStar®Plus (BMG LABTECH, Germany). The absorbance at 600 nm was measured for 60 cycles of 1 hour (24 flashes/cycle) with continual double orbital shaking (400 rpm) between measurements.

Construction of deletion mutants

CRISPR-Cas9 was used to construct *K. marxianus* deletion mutants by introduction of a repair fragment as previously described (Rajkumar & Morrissey, 2022). That protocol describes the detailed construction of plasmids to create a double stranded break (DSB) in a target gene and a repair fragment that integrates at this site to introduce a deletion that inactivates the gene. Each plasmid along with the repair fragment that

introduced a deletion of between 500 and 1500 bp (depending on the gene) was transformed into *K. marxianus* NBRC1777 *Δlig4* (Table 1) using the LiAc/SS carrier DNA/PEG method (Gietz & Schiestl, 2007). Transformant strains were checked by PCR with diagnostic primers and DNA sequencing (Eurofins Genomics, Germany) to confirm the deletion in the target locus. The primers used for plasmid construction, verification and amplifications are listed in supplementary table 1. Further details of each mutant are provided in supplementary material 1.

Gene expression analysis

For gene expression analysis, batch growth was performed in 100 mL flasks containing 25 mL of MM supplemented with the appropriate nitrogen source. The cultures were inoculated at initial A_{600} ~0.05 and the cells were collected by centrifugation at exponential phase when the A_{600} reached approximately 0.8. The pellets were immediately snap-frozen in liquid nitrogen and RNA was extracted with the Trizol method (Chomczynski & Sacchi, 1987) using glass beads as described by Tesnière et al., (2021). RNA was purified with the RNeasy Mini Kit (QIAGEN) following the RNA clean-up protocol, using DNase I for DNA digestion to remove the contaminating DNA present in the samples. The purified RNA quality was evaluated through capillary electrophoresis in the Bioanalyzer 2100 with an RNA 6000 Nano LabChip Kit (Agilent Technologies, Santa Clara, CA, USA) and quantified using a Nanodrop spectrophotometer. All the samples were diluted to the same RNA concentration at 160 ng/µL. cDNA synthesis was performed using the SuperScript III Reverse Transcriptase Kit (Invitrogen), with 100 ng/µL of random primers and 5 µL of RNA for a total reaction volume of 20 µL. The cDNA synthesis was also done for all samples without the Reverse Transcriptase III (-RT) to use as control. After this step, the cDNA samples were used as template for Real-Time qPCR with technical triplicates. The qPCR reaction of 25 µL final volume, contained 5 µL of template cDNA, 0.3 pmol/µL of each primer and 12.5 µL 2X Power SYBR Green I PCR Master Mix (Applied Biosystems, Warrington, UK). The thermocycling conditions were

as follows: an initial enzyme activation of 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of denaturation for 15 seconds at 95°C, annealing and extension for 1 minute at 60°C, with a final melt gradient starting from 15 seconds at 95°C, 30 seconds at 60°C and finally 15 seconds at 95°C. The real-time qPCRs were carried out in a 7300 Fast Real Time PCR System (Applied Biosystems). Primer specificity was confirmed by analysing dissociation curves of the PCR amplification products. The primers used for the amplification of BAT1-Long, BAT1-Total, and ACT1 mRNA are listed in supplementary table 1. Standard curves were done to calculate the primer efficiency of each primer set using genomic DNA of *K. marxianus NBRC1777* extracted with (LiOAc)-SDS method adapted from (Lõoke, Kristjuhan, & Kristjuhan, 2011). The data was normalized against *ACT1* used as control. The relative gene expression was calculated with Pfaffl mathematical model that determines the relative expression ratio (R) of the target genes based on the primer efficiency (E) and the Ct deviation of the genes of interest versus the *ACT1* control (Pfaffl, 2001). The results were plotted and significant differences between the mutants and conditions were calculated using the two-way analysis of variance (ANOVA) in GraphPad Prism. The differences were considered statistically significant when the p-values were <0.001.

Heterologous expression of fusion proteins

Three plasmids were constructed for the protein localisation experiments: pBAT1-L, pBAT1-S, pNatProm-BAT1 (Supplementary Figure 1). For the pBAT1-L and pBAT1-S construction, the BAT1 short and long open reading frame fragments were amplified by PCR from *K. marxianus NBRC1777* gDNA using Q5 polymerase (New England Biolabs (NEB) Inc., MA, USA). The fragments were then cloned into pMTU-DO-G418-C5 (Table 2) with Golden Gate Assembly, together with the GFP open reading frame part and the *TEF1* promoter (Rajkumar & Morrissey, 2020). The link between *BAT1* and GFP was 9 nucleotides long and encoded phenylalanine, tryptophan and glycine amino acids designed to form an in-frame fusion. For the construction of

pNatProm-BAT1, the fragments used were the *BAT1* native promoter that consists of 1000 bp upstream of the initiator AUG including the N-terminal extension (NTE), obtained by PCR from *K. marxianus* gDNA, and the GFP fragment with the *INU* terminator obtained by PCR from the pBAT1-S plasmid previously constructed. These fragments were cloned with the pMTU-DO-G418-C5 backbone using Gibson Assembly Master Mix (NEB) following the manufacturer's instructions. The assemblies were transformed into *E. coli* and then plated in LB medium supplemented with kanamycin 50 ng/µL. The plasmids were extracted and verified with HindIII, BstEII, NruI enzymatic digestion (NEB) and DNA sequencing (Eurofins Genomics, Germany). The resulting plasmids were transformed into *K. marxianus* NBRC1777 using the LiAC/SS carrier DNA/PEG procedure. The transformed strains were plated onto YPD agar supplemented with G418 200 ng/µL.

Fluorescence microscopy

Cultures of *K. marxianus* NBRC1777 carrying the pBAT1-L, pBAT1-S, pNatProm-BAT1 plasmids were grown at 28°C in 25 mL of MM with either ammonium or VIL as the nitrogen source at the mentioned concentrations. Cells carrying pNatProm-BAT1 plasmid were additionally grown in YPD in the same conditions. The cells were collected at exponential phase by centrifugation and resuspended in pre-warmed staining solution that consist of MitoTracker Red CMXRos (Invitrogen) at 200 nM. The suspensions were incubated for 45 minutes in the same growth medium as above and then washed with fresh pre-warmed 0.9% NaCl saline solution. The fixation was done in pre-warmed growth medium containing 3.7% formaldehyde at 37°C for 15 minutes. Antifade reagent Concanavalin-A (Invitrogen) at 0.5 mg/mL was used as mounting media for visualization. For fluorescence detection, GFP and MitoTracker Red were excited by 488 nm argon and 543 nm helium/neon lasers respectively. All images were captured using an Olympus Fluoview 1000 Confocal Laser Scanning Microscope (Olympus Corporation, Japan) with Fluoview software, using a 100x apochromatic objective with immersion oil.

Bioinformatic and statistical analysis

Expression of genes at specific genetic loci (BAT1, ALT1, BAP3) was analysed in the *K. marxianus* GWIPS-viz database (Fenton, Kiniry, et al., 2022). The prediction of the presence of a mitochondrial targeting signal (MTS) in Bat1p and the corresponding cleavage site (CS), was done with TargetP 2.0 software (Armenteros et al., 2019). The analysis of the growth kinetics data was performed using R Studio software, version 1.3.1093 (RStudio Team., 2020) with GrowthCurver package (Sprouffske & Wagner, 2016). T graphs and statistical tests were performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, California, USA). The *K. marxianus BAT1* promoter analyses was performed using the web based software YetFasco (De Boer & Hughes, 2012) and the Yeastract database (Monteiro et al., 2020) to search for putative TF-binding sites.

Data Availability

Strains and plasmids are available on request. All databases uses in this study are freely accessible: GWIPS-viz genome browser (https://riboseq.org/); Yeastract (http://www.yeastract.com/); YetFasco (http://yetfasco.ccbr.utoronto.ca/). All software listed in the relevant sections is freely available and described in the cited publications. No new code was written for this study. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables, including the supplemental material provided.

Table 1. Yeast strains used in this study. Coordinates of the mutations in the *K. marxianus* NBRC1777 genome are shown. The deleted nucleotides are represented by Δ in cases where several nucleotides were deleted.

Strain name	Genotype of mutants	Source
Kluyveromyces marxianus NBRC1777	Wild type	NITE Biological Research Centre
K. marxianus NBRC1777 Δlig4	Lig4-1 Frameshift	(Rajkumar, Varela, Juergens, Daran, & Morrissey, 2019)
K. marxianus NBRC1777 Δgat1	CHR VI Δ635,534 – 636,959	This study
K. marxianus NBRC1777 Δgcn4	CHR V Δ868,076 – 868,697	This study
K. marxianus NBRC1777 Δgln3	CHR III Δ1,456,475 – 1,457,877	This study
K. marxianus NBRC1777 Δhap2	CHR III Δ1,550,967 – 1,551,414	This study
K. marxianus NBRC1777 Δmot3	CHR VI Δ700,804 – 701,788	This study
K. marxianus NBRC1777 Δnrg1	CHR IV Δ283,460 – 284,113	This study
K. marxianus NBRC1777 Δput3	CHR VI Δ343,875 – 345,730	This study
K. marxianus NBRC1777 Δleu3	CHR VI Δ946,030 – 947,790	This study
K. marxianus NBRC1777 Δbat1	CHR I Δ1,068,093 – 1,069,223	This study
K. marxianus NBRC1777 ΔNTE-Bat1	CHR I Δ1,068,052 – 1,068,127	This study

Table 2. Plasmids used in this study

Plasmid	Source
pUDP002-HH-BsaI	(Varela, Puricelli, Montini, & Morrissey, 2019)
pMTU-DO-G418-C5	(Rajkumar & Morrissey, 2020)
pBAT1-L	This study
pBAT1-S	This study
pNatProm-BAT1	This study
pCRISPR-KmΔgat1	This study
pCRISPR-KmΔgcn4	This study
pCRISPR-KmΔgln3	This study
pCRISPR-KmΔhap2	This study
pCRISPR-KmΔmot3	This study
pCRISPR-KmΔnrg1	This study
pCRISPR-KmΔput3	This study
pCRISPR-KmΔNTEBAT1	This study
pCRISPR-KmΔbat1	This study

RESULTS

K. marxianus BAT1 has two transcription start sites (TSS)

To investigate the mechanism by which mitochondrial and cytosolic isoforms of Bat1 could arise from a single gene in *K. marxianus*, we analysed the *BAT1* locus in the recently published *K. marxianus* GWIPS-viz database (Fenton, Kiniry, et al., 2022). It was possible to identify transcription start sites (TSS-

seq), the transcribed region (RNA-Seq) and the translated region (Ribo-Seq) (Figure 1). The two tall histogram peaks in the TSS-seq panel indicate discrete transcription start sites and the RNA-Seq reads show transcription across all this region. There are two possible in-frame translation initiation codons and, from the ribosome profiling data, it is clear that translation can initiate from the first AUG codon. Since the second identified transcription start is downstream of the first AUG codon, a transcript arising from use of this TSS could only initiate translation from the second putative AUG initiation codon. Taking these data together, we hypothesised that two BAT1 mRNA isoforms are produced in *K. marxianus* through the use of alternative TSS (aTSS), each of which may be translated from a different AUG start codon. This would give rise to two different proteoforms that we refer to as Bat1-L (long) and Bat1-S (short). To test if the two versions of Bat1p are predicted to be targeted to different organelles, the subcellular localisation of the two isoforms was analysed using the in silico TargetP 2.0 tool. The program predicted the presence of a MTS in the long isoform of *BAT1*, while no signal peptide was detected in the short isoform (Figure 2). This is in agreement with the prediction of an MTS in this protein by Fenton et al., (2022b). Notwithstanding the presence of two transcriptional starts, an alternative explanation for two isoforms would be leaky scanning, which could lead to bypass of the first AUG. To assess whether this was likely, we examined the context of both AUG start codons (Figure 2). In fact, both have good Kozak consensus, largely excluding the leaky scanning hypothesis. In combination, these data support a model whereby use of alternative transcription start sites, leads to mRNA isoforms that, when translated, give rise to a longer form of Bat1 (Bat1-L) that is localised to the mitochondrion, or a shorter from of the protein that remains cytosolic (Bat1-S).

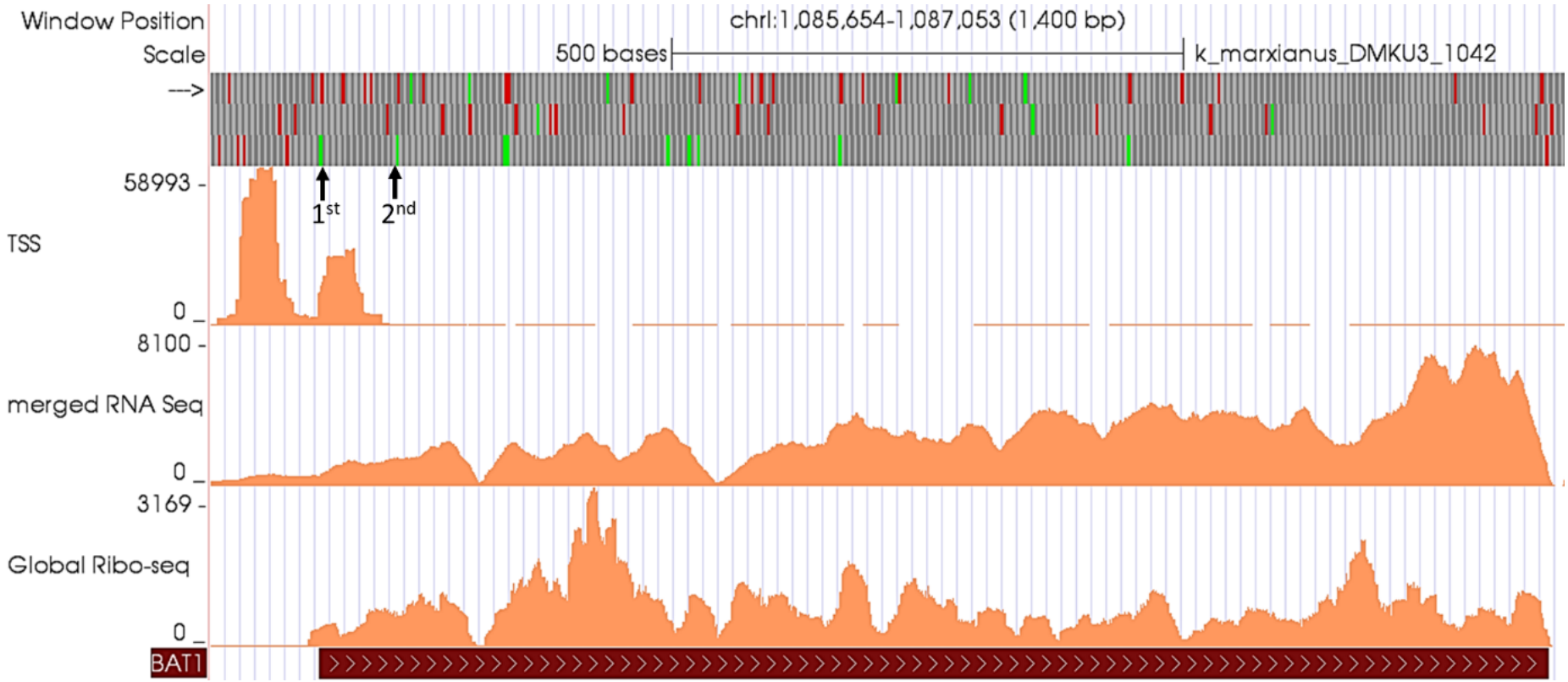

Figure 1. *K. marxianus BAT1* locus visualised on the GWIPS-Viz Genome Browser. The annotation track (red bar) at the bottom represents the coding region of *BAT1*. Coverage tracks of Ribosome profiling (Ribo-Seq), RNA Sequencing (RNA-Seq) and Transcription Start Site Sequencing (TSS-Seq) corresponding to the *BAT1* locus of *K. marxianus* are depicted. The top track represents ORF architecture including start codons in green and stop codons in red. The two black arrows indicate the positions of the first and the second in-frame start codons of *BAT1*. There are two clear TSS at the TSS-Seq track that are located upstream of each start codon.

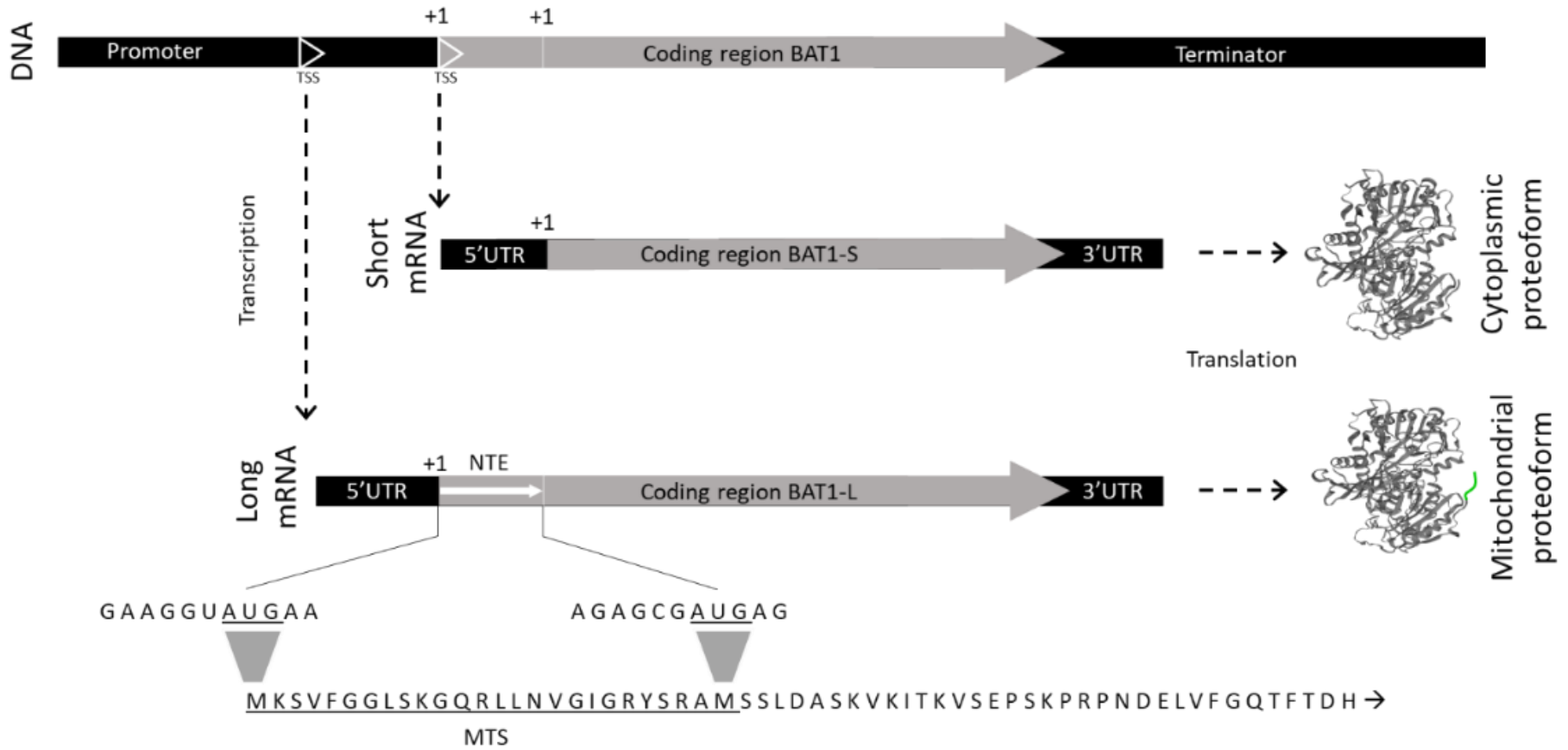

Figure 2. Diagrammatic representation of the production of two Bat1 proteoforms by the single gene. In *K. marxianus BAT1* gene is transcribed into two different mRNAs from two Transcription Start Sites (TSS). The long mRNA (BAT1-L) contains a N-terminal extension (NTE) sequence that is a putative mitochondrial targeting signal (MTS), so BAT1-L encodes a mitochondrial protein. The short mRNA (BAT1-S) encodes an identical polypeptide that lacks the MTS, producing the cytosolic proteoform. The translation initiation codons are represented as +1.

Localisation of Bat1p depends on the presence of the mitochondrial targeting signal

To understand whether the presence of the MTS determines the localisation of Bat1, fusions of the N-terminal region of Bat1-L and Bat1-S with *GFP* were made and the localisation tested when cells were grown in minimal medium with either ammonium or BCAA (a mixture of valine, isoleucine and leucine (VIL)) as the nitrogen source. For this experiment, expression was from a constitutive promoter, therefore any effects should be independent of growth medium. Cells were treated with the fluorescent probe MitoTracker red and then examined by fluorescence microscopy to visualise of mitochondria and the Bat-GFP fusions (Figure 3). In the figure, mitochondria stain red, Bat1-GFP fusions stain green, and overlap is seen as an orange/yellow colour in the merged images. It was found that Bat1-L-GFP localised to the mitochondria in both nitrogen conditions whereas only cytosolic localisation was seen for Bat1-S-GFP. These data demonstrate that the Bat1 MTS is able to localise a protein to the mitochondrion. We next evaluated whether transcripts from the native promoter of *BAT1* (pNatProm-BAT1) would encode proteins that localised to one or both cellular compartments in a nitrogen-dependent way. For this, we made a construct that included an extended *BAT1* promoter fused to *GFP* such that transcription was possible from either of the two TSS that we had identified and, depending on which TSS was used, an in-frame BAT1-GFP fusion with, or without, the MTS would be made. Localisation was determined by fluorescence microscopy under three different nutrient conditions (Figure 4). First, looking at growth with NH_4 as the nitrogen source it is seen that BAT1-GFP localises to both the cytosol and the mitochondria. This result was unexpected since it had been predicted that under these biosynthetic conditions, only mitochondrial localisation would be seen. In contrast, and in line with predictions when growing on BCAA, localisation was exclusively to the cytosol. A similar result was seen when cells were growing in the rich medium, YPD, with no mitochondrial localisation. Correlating these data to the potential use of aTSS, it suggests that when amino acids are available as

the nitrogen source (BCAA/YPD), only the shorter form of BAT1 is made (using the second TSS, whereas when there is a requirement to synthesise amino acids (NH_4), both TSS are used and the cell makes the long (mitochondrial) and short (cytosolic) forms of Bat1.

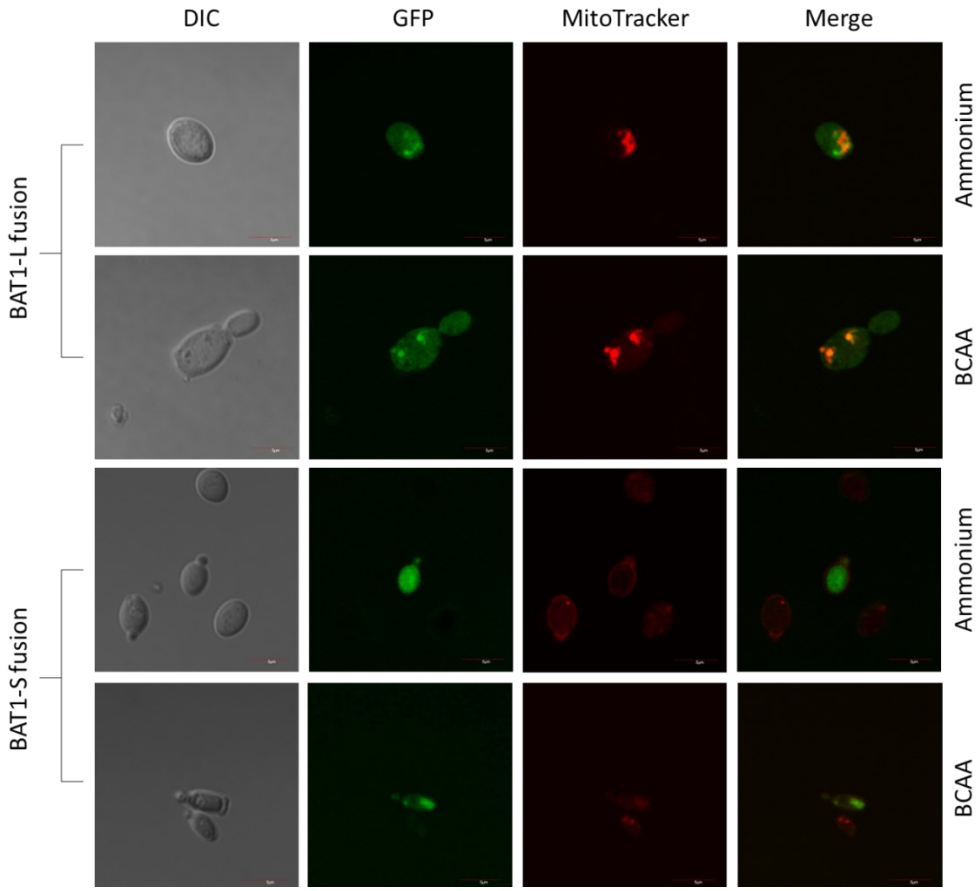

Figure 3. The mitochondrial targeting sequence (MTS) of Bat1 is responsible for mitochondrial localization. Cells transformed with BAT1-L and BAT1-S GFP fusions expressed from the constitutive *TEF1* promoter were grown on ammonium or BCAA, stained with MitoTracker Red and imaged with fluorescence microscopy. The first two rows correspond to cells transformed with BAT1-L fusion carrying the MTS and the second two rows correspond to cells transformed with BAT1-S. From left to right are the images from Differential Interference Contrast (DIC) light, Green Fluorescence Protein (GFP) fluorescence, MitoTracker fluorescence and the merge of GFP and MitoTracker fluorescence. Orange/yellow fluorescence merged images is seen in cases of co-localisation.

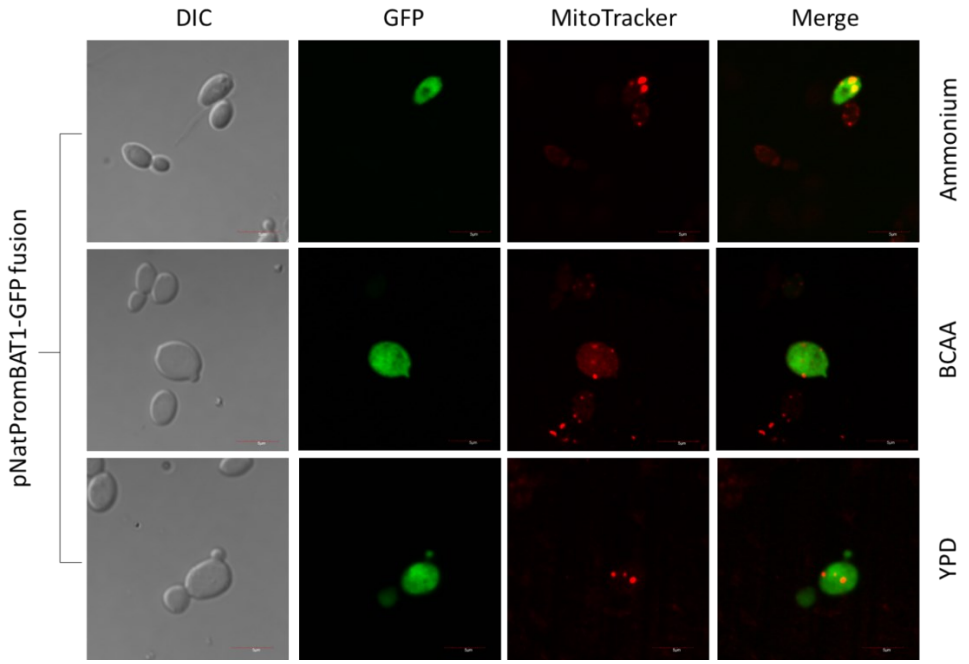

Figure 4. The *BAT1* promoter determines localisation of NTE-GFP in *K. marxianus*. Cells transformed with the pNatProm-BAT1-GFP fusion were grown on ammonium, BCAA or YPD, stained with MitoTracker Red and imaged with fluorescence microscopy as described in Figure 3. From left to right are the images from Differential Interference Contrast (DIC) light, Green Fluorescence Protein (GFP) fluorescence, MitoTracker fluorescence and the merge of GFP and MitoTracker fluorescence. Orange/yellow fluorescence is seen when the NTE-GFP protein localises in the mitochondria (Ammonium).

Expression of different *BAT1* isoforms depending on the nitrogen conditions

To explore the proposed use of aTSS under different nitrogen conditions, the wild-type strain was grown in ammonium and VIL separately and BAT1 mRNA transcripts quantified by RTqPCR. One pair of primers within the *BAT1* open reading frame allowed quantification of the total amount of BAT1 mRNA – BAT1-Total. A second pair specifically amplifies the longer mRNA isoform – BAT1-L, and the levels of BAT-S deduced from the difference between the total amount of BAT1 mRNA and the level of BAT-L. Constitutively expressed *ACT1* was used as a reference and the relative levels of the different isoforms determined when the cells were growing on either ammonium or VIL as a nitrogen source (Figure 5). The overall amount of BAT1 mRNA present in cells growing on VIL was approximately 60% of that

seen on ammonium but the major differences arose when comparing the relative amounts of the two isoforms. When growing on ammonium, there were approximately equal amounts of both isoforms whereas on VIL, the shorter isoform (BAT1-S) was almost exclusively present. These data indicate that when growing on BCAA, only the second TSS is used, whereas on ammonium, both TSS seem to be used in approximately equal measure.

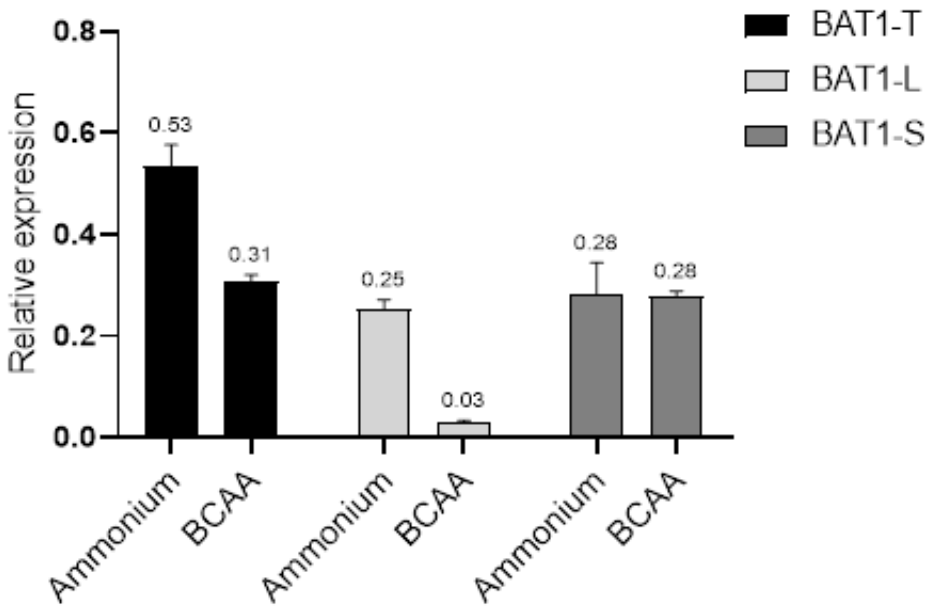

Figure 5. Expression the *BAT1* long isoform (BAT1-L) is decreased when cells are grown on BCAA. Levels of BAT1-L and total BAT1 (BAT1-T; that includes both the long and short isoforms) were measured by RT-qPCR from *K. marxianus* grown on ammonium and BCAA (valine, isoleucine and leucine) and used to also determine the level of BAT-S. The gene expression values are relative to the expression of *ACT1*. Error bars were calculated from three independent experiments with technical triplicates.

Role of the transcription factors in the expression of the long isoform

We next sought to identify the transcriptional factors (TFs) responsible for the use of aTSS in *BAT1*. Two approaches were taken to generate a list of candidate TFs. First, we included TFs that had been shown to be involved in regulation of either *BAT1* or *BAT2* in *S. cerevisiae* (González et al., 2017). Second, we examined the *BAT1* promoter using the *S. cerevisiae* consensus and found potential binding sites that fully matched the consensus for Gcn4,

Gln3/Gat1, Hap2, Mot3 and Nrg1, and that deviated by only one nucleotide for Leu3 and Put3. Using a CRISPR-Cas9 method, we created eight deletion mutants, each lacking one of these TFs. The mutants were grown in ammonium and VIL separately and the levels of total BAT1 mRNA and BAT1-L were determined to establish which, if any, of these TFs played a role in expression (Figure 6). For this analysis, the BAT1 mRNA levels in each mutant was compared to that of the wild-type growing under the same conditions and there were clear indications of the involvement of multiple TFs. Looking first at ammonium, it is seen that deletion of *GCN4* had the greatest effect. The total amount of BAT1 mRNA was reduced by about 50%, with essentially none of the longer form made (BAT1-L). Given the earlier data that there is approximately equal transcription from both the first and the second TSS under these conditions (Figure 5), this result indicates that Gcn4 is required for transcription from the first TSS but not the second. Deletion of *LEU3* led to a 50% reduction in BAT-L, showing that Leu3 activates expression from the first TSS but it is not absolutely required. On BCAA, there were visible increases in the total amount of BAT1 in some mutants, though this did not pass the threshold for statistical significance. Looking specifically at use of the first TSS however, a substantial increase in the amount of the long isoform was seen in three mutants: *put3, nrg1* and *hap2.* Although fold-increases were high, they did not dramatically change the total amount of BAT1 mRNA because the level of BAT1-L was coming from a very low base in the wild-type (Figure 5). Nevertheless, the conclusion is that all three TFs impair expression from the first TSS on BCAA. Since most *BAT1* expression on BCAA is of the short isoform, these data suggest that none of these TFs are required for expression from the second TSS on BCAA.

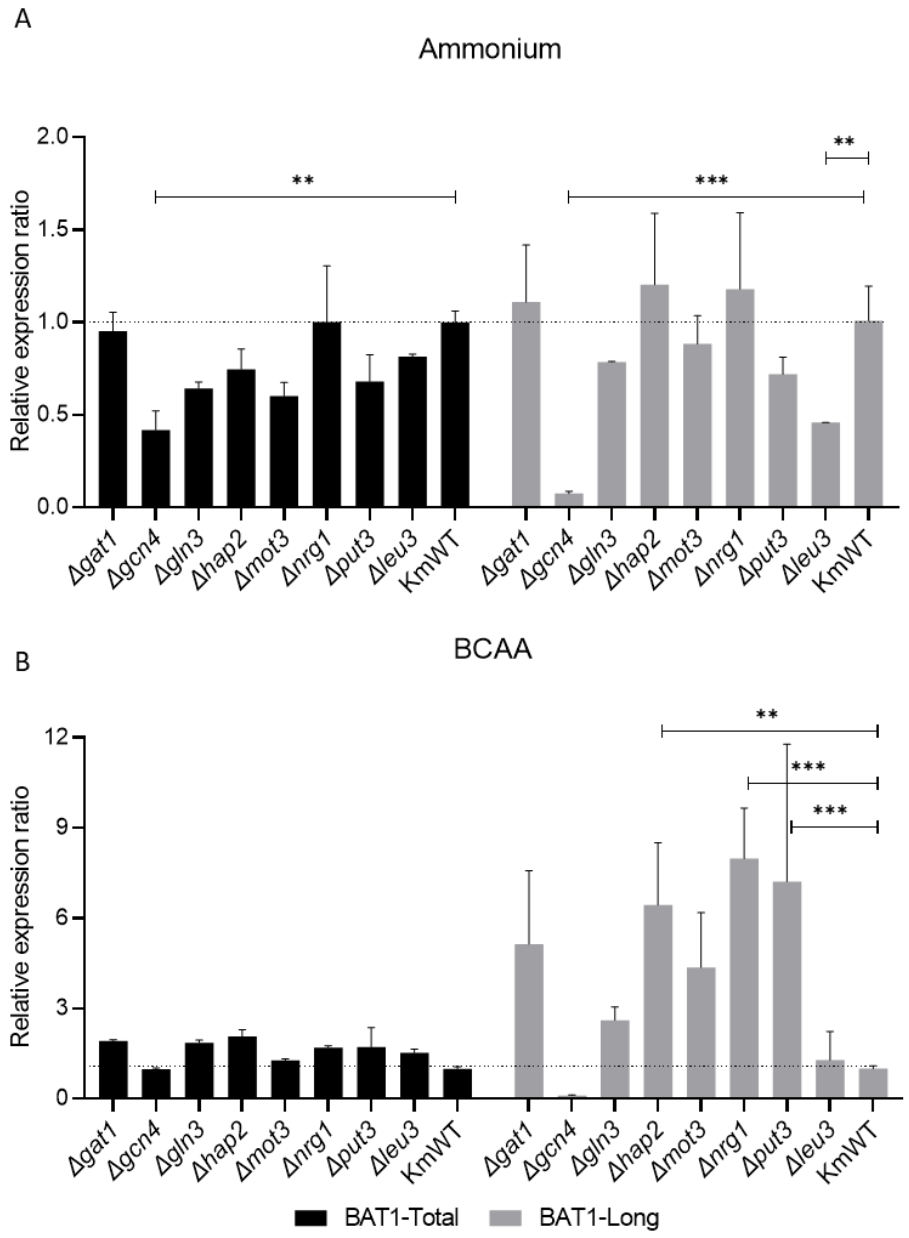

Figure 6. Role of transcription factors in the expression of *BAT1*. Levels of BAT1-L and total BAT1 (BAT1-T; that includes both the long and short isoforms) were measured by RT-qPCR from the indicated *K. marxianus* wild-type (WT) and mutant strains grown on ammonium (panel A) and BCAA (valine, isoleucine and leucine)(panel B). Data are normalised to ACT1 mRNA levels and the ratio of BAT1-L and BAT1-T in each mutant relative to wild type is shown. Data represent two independent experiments with technical triplicates. The differences were considered statistically significant when the p-values were <0.001 (***) or <0.01 (**).

Function of Bat1p in *K. marxianus* depending on the presence of the NTE

The data are consistent with the hypothesis that when growing on ammonium (biosynthetic conditions), both proteoforms are made, with the longer form localising to the mitochondrion to enable synthesis of BCAA. On VIL as a nitrogen source (catabolic conditions), the mitochondrial proteoform would not be required and cytosolic Bat1 is involved in degradation of the BCAA to provide nitrogen for the cell. To explore this further, we constructed two mutants of *BAT1*, one completely inactivating the gene (*Δbat1*) and one precisely deleting the MTS so that only the cytosolic proteoform is made (*ΔNTE-bat1*). These mutants were then grown on different sole nitrogen sources and the maximum specific growth rates (μ_{max}) were calculated (Figure 7). The *Δbat1* mutant showed severely impaired growth on biosynthetic conditions (ammonium and glutamine) and on individual amino acids, but only moderately impaired growth in catabolic conditions (BCAA). When provided with both ammonium and BCAA, growth of *Δbat1* was restored almost to the wild-type rate confirming that Bat1 is required for both the anabolism and catabolism of BCAA. Somewhat unexpectedly, the *ΔNTE-bat1* strain did not display any growth impairment on ammonium, indicating that BCAA biosynthesis is possible even in the absence of the mitochondrially-targeted protein. In fact, only discernible phenotype for *ΔNTE-bat1* was a reduction of about 50% in growth rate on BCAA.

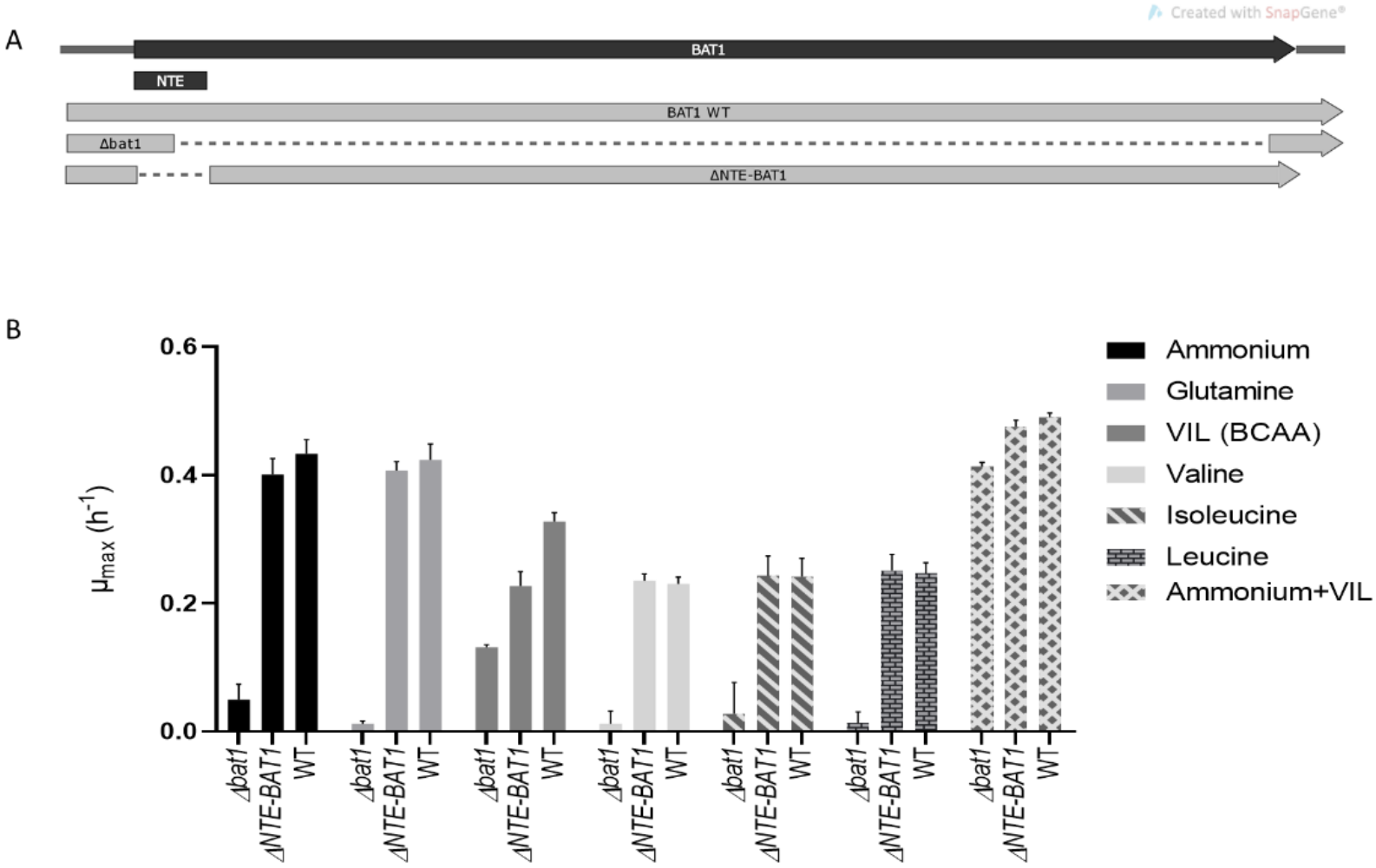

Figure 7. Growth rates of *K. marxianus* bat1 mutant strains. Two mutant strains, one lacking the B*AT1* coding region (Δ*bat1*) and one with a precise in-frame deletion of the mitochondrial targeting sequence (ΔNTE-*BAT1*) were generated and growth rates on different nitrogen sources were determined. (A) Schematic depiction of the *K. marxianus* mutants. (B) Bar graph of maximum specific growth rate (μ_{max}) of mutants and wild type (WT) grown in the indicated nitrogen sources. Data were compiled from four independent experiments with technical replicates.

Wider use of aTSS for controlling sub-cellular localisation

The data with *BAT1* raise the question as to whether the mechanism of use of alternative TSSs for the production of two protein isoforms with different sub-cellular localisation might be more widespread. To explore this, we used the GWIPs platform to examine two other *K. marxianus* genes, *ALT1* and *BAP3*, that may also need to encode differentially located proteoforms. In *S. cerevisiae*, *ALT1/ALT2* (Peñalosa-Ruiz et al., 2012) and *BAP2/BAP3* (Regenberg, Düring-Olsen, Kielland-Brandt, & Holmberg, 1999) are paralogous pairs, one of which encodes a mitochondrially-localised proteoform. In both cases, we found that the *K. marxianus* orthologue has more than one TSS and a second in-frame AUG (Figure 8). Under the conditions that the data was generated, there is unambiguous translation from first AUG (based on the ribosome profiling footprints), but the major transcription start site is downstream of this. The most straightforward explanation is that, as with *BAT1*, alternative transcription starts sites are used to generate mRNAs that give rise to proteins with alternative N-termini. For *ALT1*, there is a relatively short N-terminal extension that encodes a canonical MTS (TargetP 2.0 score 0.993), and it is clear that differential use of the aTSS can give rise to the alternative proteoforms that will differentially localise in the exact same manner as with *BAT1*. *BAP3* is somewhat different since the difference in length between the two proposed proteoforms is 71 amino acids and an MTS is not evident in the N-terminal extension. Bap2/Bap3 proteoforms localise to the plasma-membrane, ER and mitochondrion in *S. cerevisiae* (Regenberg et al., 1999), and it is possible that the localisation uses an alternative mechanism (such as protein folding) to the canonical MTS. Although more experimental data would be required to elucidate details, taking *BAT1, ALT1* and *BAP3* as examples, it can be inferred that the use of aTSS to direct the synthesis of differentially-localised proteoforms is common.

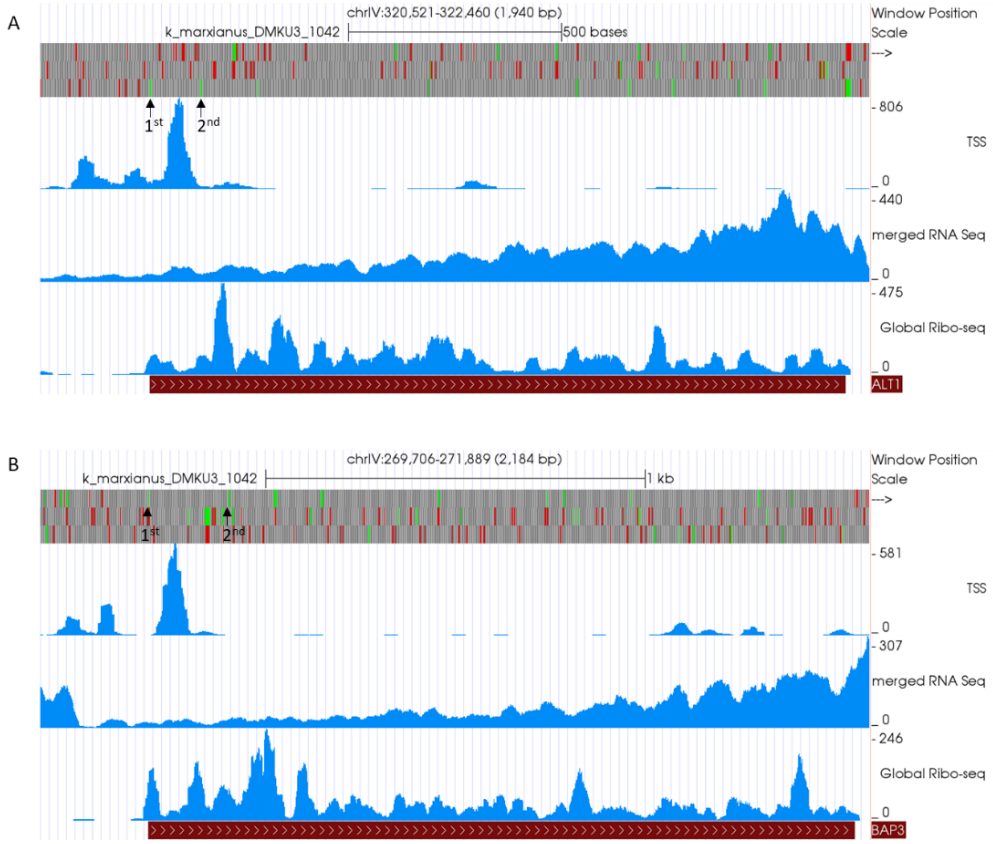

Figure 8. Visualisation of the *ALT1* and *BAP3* loci in the *K. marxianus* GWIPS-Viz Genome Browser. Panel 1, *ALT1*; panel B, *BAP3.* The red bar at the bottom represents the coding region of each gene and the top tracks depicts the ORF architecture including start codons in green and stop codons in red (*ALT1*, 3rd track; *BAP3*, 1st track). Note that scales are not identical. The two black arrows indicate the positions of the first and the second in-frame start codons of each gene Coverage of Ribosome profiling (Ribo-Seq), RNA Sequencing (RNA-Seq) and Transcription Start Site Sequencing (TSS-Seq).

DISCUSSION

The increased availability of omics datasets and on-line databases for non-traditional or non-conventional yeasts are valuable assets for the investigation of the evolution of gene regulatory mechanism. We exploited these resources to perform an *in silico* analysis of the gene encoding branched chain aminotransferases (BCAT) in *K. marxianus*, a species that diverged from the *Saccharomyces* lineage prior to the whole genome duplication (WGD). Previous studies in the related species *K. lactis* and *L. kluyveri* had established that *BAT1* is bifunctional, encoding both the mitochondrial and cytosolic BCAT, required for synthesis or degradation of branched chain amino acids (BCAA), respectively. It was proposed that

synthesis of the required proteoform was dependent on the nitrogen source in the growth medium, but the underlying mechanism was not determined. In principle, there are a number of different means that yeast could use to generate these alternative proteoforms. For example, a global analysis of the transcriptional and translational landscape in *K. marxianus* found that proteins with alternative N-termini could be generated through the use of alternative transcriptional start sites (aTSS) or leaking scanning of a single transcript, whereby an AUG in a poor context (or a non-cognate start codon) is bypassed enabling initiation at a downstream AUG codon (Fenton et al., 2022b). Another system is seen with the *S. cerevisiae* fumarase Fum1, which uses alternative protein folding to control the distribution of this enzyme between the mitochondrion and cytosol (Herrmann, 2009; Regev-Rudzki, Battat, Goldberg, & Pines, 2009). By analysing transcription start sites (TSS), transcriptome data, and ribosome profiling data, we formed a hypothesis that *K. marxianus* uses aTSS to produce cytosolic or mitochondrial proteoforms of BCAT. We proposed that when biosynthesis of BCAA was required, transcription would initiate at the first TSS giving rise to a longer proteoform containing a mitochondrial targeting signal (MTS), whereas when BCAA are in excess or providing the cell with a source of nitrogen, the second TSS would be used, producing a shorter cytosolic proteoform. Using GFP fusions, we confirmed both the presence of an MTS and nitrogen-source differential localisation of Bat1. Finally, we directly measured levels of mRNA isoforms that would arise from use of the first or the second TSS and found that when the yeast is growing using BCAA as a N-source, there is essentially no transcription from the first TSS – only the second TSS is used and therefore only the cytosolic proteoform is made. In contrast, under biosynthetic conditions (NH_4 as a N-source), both transcription start sites are used in almost equal measure leading to synthesis of mitochondrial and cytosolic proteoforms.

Having established that synthesis of the proteoforms is controlled at a transcriptional level, we used a combination of *in silico* analysis of the *BAT1*

promoter and knowledge from *S. cerevisiae* to identify transcription factors (TFs) that could be responsible for the transcriptional control. Eight candidate TFs were individually inactivated by CRISPR-Cas9 – mediated deletion and the levels of the mRNA isoforms measured under different conditions. The strongest effect was seen for Gcn4, which appears to be absolutely required for transcription from the first TSS when the cells are growing under biosynthetic conditions (NH_4). The *K. marxianus BAT1* promoter harbours the consensus binding sequence of Gcn4p ~59 bp upstream the first TSS, ideally located for direct binding of Gcn4 to drive expression. This accords with data from *S. cerevisiae* where it has long been known that Gcn4 is a transcriptional activator of amino acid biosynthetic genes, responding to amino acid starvation by a translational control mechanism (Hinnebusch, 2005; Natarajan et al., 2001). Furthermore, in *S. cerevisiae*, Gcn4 directly binds to the *BAT1* promoter and activates expression only in biosynthetic conditions (Gonzalez et al., 2017), a situation that is analogous to transcription of the longer mRNA isoform in *K. marxianus*. Although, less pronounced, Leu3 is also required for full activation of expression from the first TSS when cells are growing under biosynthetic conditions (NH_4). This also mirrors *S. cerevisiae* where Leu3 activates expression of *BAT1*, though in this case, Leu3 is considered the major activator. The candidate Leu3 binding sites in the *K. marxianus BAT1* promoter deviate by a single nucleotide from the consensus and are quite distal from the transcription start site. One or both of these factors may explain the apparent difference in importance of Leu3 in the two yeasts.

Looking at catabolic conditions (BCAA), we found roles for three TFs. Interestingly, none of these was a positive effect since the data indicate that Put3, Nrg1 and Hap2 inhibit transcription from the first TSS on VIL. Nrg1 works by recruiting the Ssn6-Cyc2 co-repression complex and it is possible that this TF represses transcription by recruiting this complex to the *BAT1* promoter (Murad et al., 2001; Park, Koh, Chun, Hwang, & Kang, 1999). Hap2 forms part of the global regulator HAP complex, binding the 5'-CCAAT-3'

sequence. This conserved binding site is found in 30% of eukaryotic genes and tends to be located at 60 to 100 bp from the TSS (Dolfini, Zambelli, Pavesi, & Mantovani, 2009) whereas in the *BAT1* promoter, it is 450 bp upstream from the first TSS. Possibly the HAP complex may interact with Nrg1 to recruit the Ssn6-Cyc2 co-repressors or, alternatively, the effect may be indirectly mediated via another gene, itself regulated by the HAP complex. In *S. cerevisiae,* under catabolic conditions, Put3 binds to the *BAT2* promoter, activating expression and is believed to also repress expression of *LEU1*, reducing the levels of α-iPM and essentially switching the role of Leu3 at the *BAT1* promoter from an activator to a repressor (González et al., 2017). The *K. marxianus BAT1* promoter has a sequence that differs from the Put3 consensus by one nucleotide so in principle Put3 binding to the promoter is possible. Interestingly, the *K. marxianus LEU1* promoter lacks a consensus Put3 binding site so it is possible that the negative effect of Put3 on transcription from the first TSS is a direct effect. In contrast to *S. cerevisiae BAT2*, there is no evidence that Put3 activates expression from the second TSS in *K. marxianus*. There is also no evidence for a role for Gln3. In *S. cerevisiae*, expression of *BAT2* involves direct binding of Gln3, the classic TF involved in the NCR system. This is not the case in *K. marxianus* since there is not a loss of transcription from the second TSS in the *gln3* mutant. Although a putative Gln3 binding site is present in the *Kluyveromyces BAT1* promoter, it is far from the transcription start sites and unlikely to be used. In fact, this is similar to the *S. cerevisiae BAT1* promoter where deletion of putative Gln3 binding sites had no effect (González et al., 2017).

One of the goals of this study was to address the hypothesis that retention of both BCAT paralogues in *S. cerevisiae* offers greater regulatory control than in the ancestral state where both proteoforms are synthesised from a single gene. Our data support that hypothesis, since we showed that while the pre-WGD form of the gene is capable of differential synthesis of the two proteoforms, the regulatory control appears to be less tight than that of the post-WGD form. This conclusion is based on multiple data showing that

under biosynthetic conditions both proteoforms are made in *K. marxianus*, while, in *S. cerevisiae*, only the mitochondrial proteoform is made. Under catabolic conditions, equal resolution is achieved. Mechanistically, the difference can be explained by the fact that it is easier to control the expression of two distinct genes than the use of two transcription start sites 75 nucleotides apart. Based on our data, we propose that the ancestral gene largely controlled expression ratios via regulation at the first TSS – either activating via Gcn4 and Leu3 or repressing via Put3 or Ssn6-Tup1 (recruited by Nrg1) (Figure 9). Most of these mechanisms were retained in *BAT1* following the WGD, though the Put3 regulon expanded to include *LEU1* offering an additional means to repress *BAT1*. The pre-WGD expression of the second TSS, giving rise to the cytosolic proteoform, was essentially unregulated but in post-WGD species, *BAT2* lost the MTS and acquired new regulation, specifically by integration into the NCR system (via Gln3) and co-opting Put3 as a second activator by optimising the sub-optimal binding sites. At the same time, the constitutive expression of this proteoform seen in *K. marxianus* was lost, therefore *BAT2* is actually dependent on these activators for expression. One interesting feature of *S. cerevisiae BAT2*, not seen in *BAT1*, is the presence of positioned nucleosomes that occlude some potential binding sites. The gene promoters in both pre- and post-WGD species have binding sites for several TFs associated with chromatin remodelling, which is suggesting of a role for chromatin structure in regulating expression. Although major effects were not seen with relevant single mutants in either *S. cerevisiae* or *K. marxianus*, it is possible that there is redundancy and this would be an interesting area to explore further. Overall, our data supports a process whereby post-WGD yeasts maintained most of the ancestral regulation in one copy of a duplicated gene and adapted existing cis and trans acting factors to develop a new regulatory framework for the second copy.

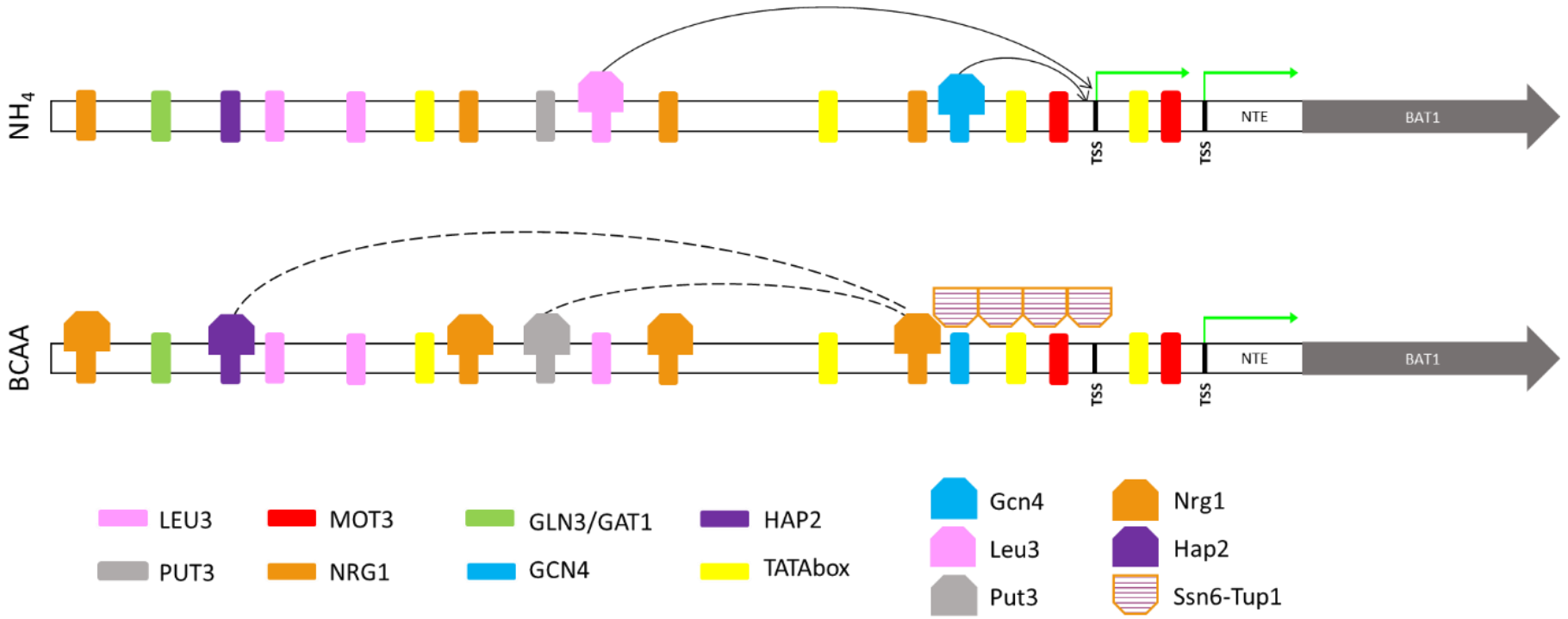

Figure 9. Cartoon with the proposed transcriptional regulation mechanism of *BAT1* in *K. marxianus* when growing under biosynthetic (NH_4) or catabolic (BCAA) conditions. Potential binding sites in the BAT promoter (~600 bases upstream of the first AUG are included) for different transcription factors (TF) are show in various colours. Transcription from the first or the second TSS is indicated with a green arrow. When growing on NH_4, it is proposed that Leu3 and Gcn4 and activate expression from the first TSS. Expression from the second TSS is also possible and so both the cytosolic and mitochondrial forms of Bat1 are made. Only binding to one putative Leu3 motif is shown for simplicity. When growing on BCAA, it is proposed that Nrg1 recruits the Ssn6-Tup1 co-repressor complex, leading to occlusion of the first TSS but allowing use of the second TSS – thereby making the cytosolic form of Bat1. It is suggested that Hap2 and Put3 may contribute to the recruitment of the co-repression complex but that is quite tentative.

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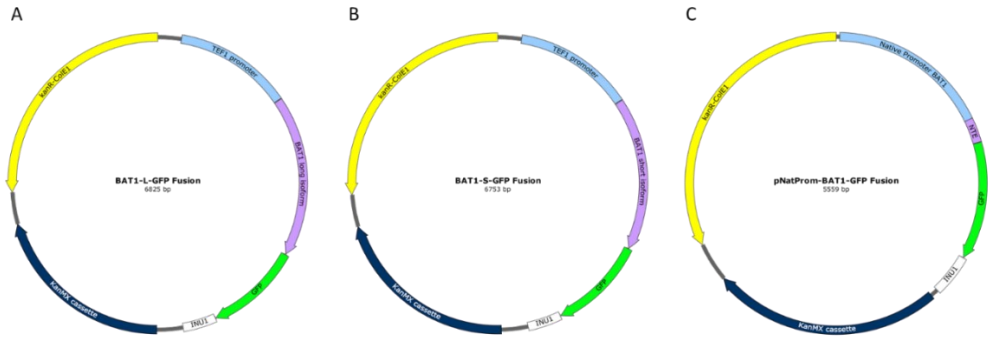
Conflict of Interest P.V.B is a co-founder of RiboMaps Ltd, a company that provides ribosome profiling as a service.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Plasmids constructed for localisation of *BAT1* in *K. marxianus*. (A) BAT1-L fusion constructed for localisation of the long isoform of *BAT1* fused with GFP and induced by *TEF1* promoter. (B) BAT1-S fusion constructed for localisation of the short isoform of *BAT1* fused with GFP and induced by *TEF1* promoter. (C) pNatProm-BAT1 fusion containing the NTE fused to GFP and induced by the endogenous promoter of *BAT1.* All plasmids have *INU1* terminator. KanMX cassette for G418 resistance used for yeast selection and KanR-ColE1 contains the replication origin and selection marker of bacteria with kanamycin.

Supplementary table 1. Primers used in this study

Primer name	Sequence (5'-3')	Experiment
BAT1-Long-F	GAACCCACAGTTATAGAGTATAGATTATTAAAAGG	qPCR
BAT1-Long-R	GACCAAATACCAATTCATCGTTTGG	
BAT1-Total-F	GAACGTAGGGATCGGAAGATATAGC	
BAT1-Total-R	GATGGGTCCAAAGAGAGGTTCC	
ACT-F	GGCTGAACGTGGTTACTCCT	
ACT-R	AGAAGCGGTTTGCATTTCTT	
SCR1-F	ATCTCGCATGGGAAAGCC	
SCR1-F	TCTGGATCCAAACGTACTCC	
GFP_F	GCATCGTCTCATCGGTCTCATTCTGGGGSAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGAT	Plasmid assembly
GFP_R	ATGCCGTCTCAGGTCTCAGGATTTAGTATAGTTCATCCATGCCATGTGTAATCCCAGCAGCTGTTACAAACTCAAGAAGGACCATGTGGTCCCTCTTT	
BAT1-Long-GFP-F	GCATCGTCTCATCGGTCTCATATGAAGAGCGTTTTCGGAGGTCTAAGC	
BAT1-Short-GFP-F	GCATCGTCTCATCGGTCTCATATGAGTTCATTGGATGCGTCAAAAGTGAA	
BAT1-GFP-R	ATGCCGTCTCAGGTCTCAAGAAGTTCAAATCAGCAACAATACGGGACC	
NativePromBAT1-Gibson2-F	CCCTGAATTCGCATCTAGACTGATTGGGGCTCCCGCAATTGGT	
NativePromBAT1-Gibson2-R	AAGTTCTTCTCCTTTACTCATCGCTCTGCTATATCTTCCGA	
GFP-INU1-Gibson2F	ATCGGAAGATATAGCAGAGCGATGAGTAAAGGAGAAGAACTT	
GFP-INU1-Gibson2-R	GGTTCGTAACATCTCTGTAACTGCTCAGCCACCTTTCTAAGGTTAGAA	
Diag-pBAT1NativeProm-F	CTCACGACGGCGATCACAG	
Diag-pBAT1NativeProm-R	CCTTCACCCTCTCCACTGACAG	
Diag-in-BAT1L-F	TTCCGTGCAGACAAGAACA	
Diag-in-BAT1L-R	CGAGCTGGGGCAAC	
CRISPR-T1-dLEU3-F	CGTCGTCTAGCTTCAGATGAAGAA	
CRISPR-T1-dLEU3-R	AAACTTCTTCATCTGAAGCTAGAC	
RF-dLEU3-F	TCAGTCACTATCAAGTTTGGGGTCGGAAGAATTACTGAAAAAACTGGATGCTGATGGAACTGTTAACAGA	

RF-dLEU3-R	AGAGATGATTTTTCTTGCATTTTCCTCCGGGTTTTTGTCCTCTGTTAACAGTTCCATCAGCATCCAGTTT	Construction of mutants
Diag-dLEU3-F	AAATACCAGTAGCGCGTCTACTCC	
Diag-dLEU3-R	TAGTTCTGAGACAATGGGCTTGGG	
CRISPR-T1-dGAT1-F	CGTCCCGGGTATAATGAACGATGG	
CRISPR-T1-dGAT1-R	AAACCCATCGTTCATTATACCCGG	
RF-dGAT1-F	GTTGAAGTTTGAAGGAGTGTCGTTTTCGTTGCAGGATTGGAAGGGTTCGGAGTTGATAATAATAATAAAA	
RF-dGAT1-R	TTAGCATTCGAGGCAATACTGTTGGCATTATTATTATTACTTTTATTATTATTATCAACTCCGAACCCTT	
Diag-dGAT1-F	GAGACCAGAGTACTCTATGCGACT	
Diag-dGAT1-R	GTGTTTGGCTGTCTATATCATTTTTTCCTTCATACG	
CRISPR-T1-dGCN4-F	CGTCCGAGTTGGACAATCAGGACT	
CRISPR-T1-dGCN4-R	AAACAGTCCTGATTGTCCAACTCG	
RF-dGCN4-F	AATGGGTGAATTGATCTTTGACAAGTTTATCAACCACGTTGTCGATCACCCAGTGTTGCATACAGCAGAA	
RF-dGCN4-R	GATTCGGGGATCACCGGAGTCAAAGGAGCTGAACGTTGCTTTCTGCTGTATGCAACACTGGGTGATCGAC	
Diag-dGCN4-F	GAAAATGAATATGAATATGAATATGAATACGAGTACAAATATGAACA	
Diag-dGCN4-R	TTCCAGCAATTCCTTGACCTTCTCT	
CRISPR-T1-dGLN3-F	CGTCACCACTTTGGAGACGTGATC	
CRISPR-T1-dGLN3-R	AAACGATCACGTCTCCAAAGTGGT	
RF-dGLN3-F	AACGTTGACGAGTTTATGATGACCCCAAGCGAACACAGCGATTCTGCTACCATTAAACCCTAATGGGAGT	
RF-dGLN3-R	AAATAGTTGCATTACTTGACGAGTGGTTTGATGTTGATAAACTCCCATTAGGGTTTAATGGTAGCAGAAT	
Diag-dGLN3-F	AAATGAACGCCCGTGGACT	
Diag-dGLN3-R	GGTGCTGCTGTTGTTGCTGTTC	
CRISPR-T1-dHAP2-F	CGTCGCCGGAATTAGTAACTACCC	
CRISPR-T1-dHAP2-R	AAACGGGTAGTTACTAATTCCGGC	
RF-dHAP2-F	CATATGCAGAACACTTTAAAATAATACAGCAATGTGTCGAGGTTTACAAATATGAAGTATCTCAAGGAAA	
RF-dHAP2-R	TTCTGCTAATTCTGGATGGAAAGAAGTAGCTAGAATTCTATTTCCTTGAGATACTTCATATTTGTAAACC	
Diag-dHAP2-F	TAGAAAAGGCTAATTTCATGTTCAAGGATTGATATCAGA	
Diag-dHAP2-R	ATCCCGTCAGTGTCAATTTTTTTGGT	

CRISPR-T2-dMOT3-F	CGTCGAGCAGTACTGAGAGTAATA	
CRISPR-T2-dMOT3-R	AAACTATTACTCTCAGTACTGCTC	
RF-dMOT3-F	TTAAACCAAGGATCGAATTCGACACCCTTGGTCATACACCCTGTTTACTACTCGACTGCCAATTTAGTAA	
RF-dMOT3-R	TCGTTATTGATTAACCACACTTTATCCCCAGTGTTGGTTGTTACTAAATTGGCAGTCGAGTAGTAAACAG	
Diag-dMOT3-F	GGATTGGGTTCGGTAACCTTATGGT	
Diag-dMOT3-R	TCACTTACACTTGTGATCCACTATGTAATTCAAACA	
CRISPR-T1-dNRG1-F	CGTCCACGACGTGCTCGTTCACGT	
CRISPR-T1-dNRG1-R	AAACACGTGAACGAGCACGTCGTG	
RF-dNRG1-F	CAGCGCCAAGGCCGATTTTACCACCATTGCATTCTGTTATTCTAAATAGCTACTCGAGGCTTCACTACCT	
RF-dNRG1-R	TCGCCAGTGTGTATTCTGTTGTGACGGGCGAGATGGCCCGAGGTAGTGAAGCCTCGAGTAGCTATTTAGA	
Diag-dNRG1-F	TCCAGGCAGCTCGAGAACG	
Diag-dNRG1-R	ACTGCGTTTGGATGGTTCTTGAAA	
CRISPR-T1-dPUT3-F	CGTCGAAAACGTCACGAAGGAAGG	
CRISPR-T1-dPUT3-R	AAACCCTTCCTTCGTGACGTTTTC	
RF-dPUT3-F	GAACGGAATACGCGACTGCAGGCTCTGGAGCTGCTCAAGAAACAATTGAGACTGGTTCACGATGACAGAA	
RF-dPUT3-R	GATGAGAATCCAGTTGATACGTGAGACACTATCTTAACATTTCTGTCATCGTGAACCAGTCTCAATTGTT	
Diag-dPUT3-F	AGAAGATGCGGAAGTTTGGGAT	
Diag-dPUT3-R	GGGCAGGATGTATCGAATCCA	
CRISPR-T1-dNTEBAT1-F	CGTCGTTTTCGGAGGTCTAAGCAA	
CRISPR-T1-dNTEBAT1-R	AAACTTGCTTAGACCTCCGAAAAC	
RF-dNTEBAT1-F	AAGAACCCACAGTTATAGAGTATAGATTATTAAAAGGTTTTTGCAATTAGAAGGTATGAGTTCATTGGAT	
RF-dNTEBAT1-R	TCGATGGCTCAGAAACCTTCGTAATTTTCACTTTTGACGCATCCAATGAACTCATACCTTCTAATTGCAA	
Diag-dNTEBAT1-F	GATGTGTCATACCCTGTGACATACCCTGT	
Diag-dNTEBAT1-R	ACTTGATCAACTCCTCCCCATCAAAC	
CRISPR-T1-dBAT1-F	CGTCGAGGAGTTGATCAAGTTGAT	
CRISPR-T1-dBAT1-R	AAACATCAACTTGATCAACTCCTC	
RF-dBAT1-F	TGCAATTAGAAGGTATGAAGAGCGTTTTCGGAGGTCTAAGCAAGGGCCAAAGGTTGGTCCCGTATTGTTG	

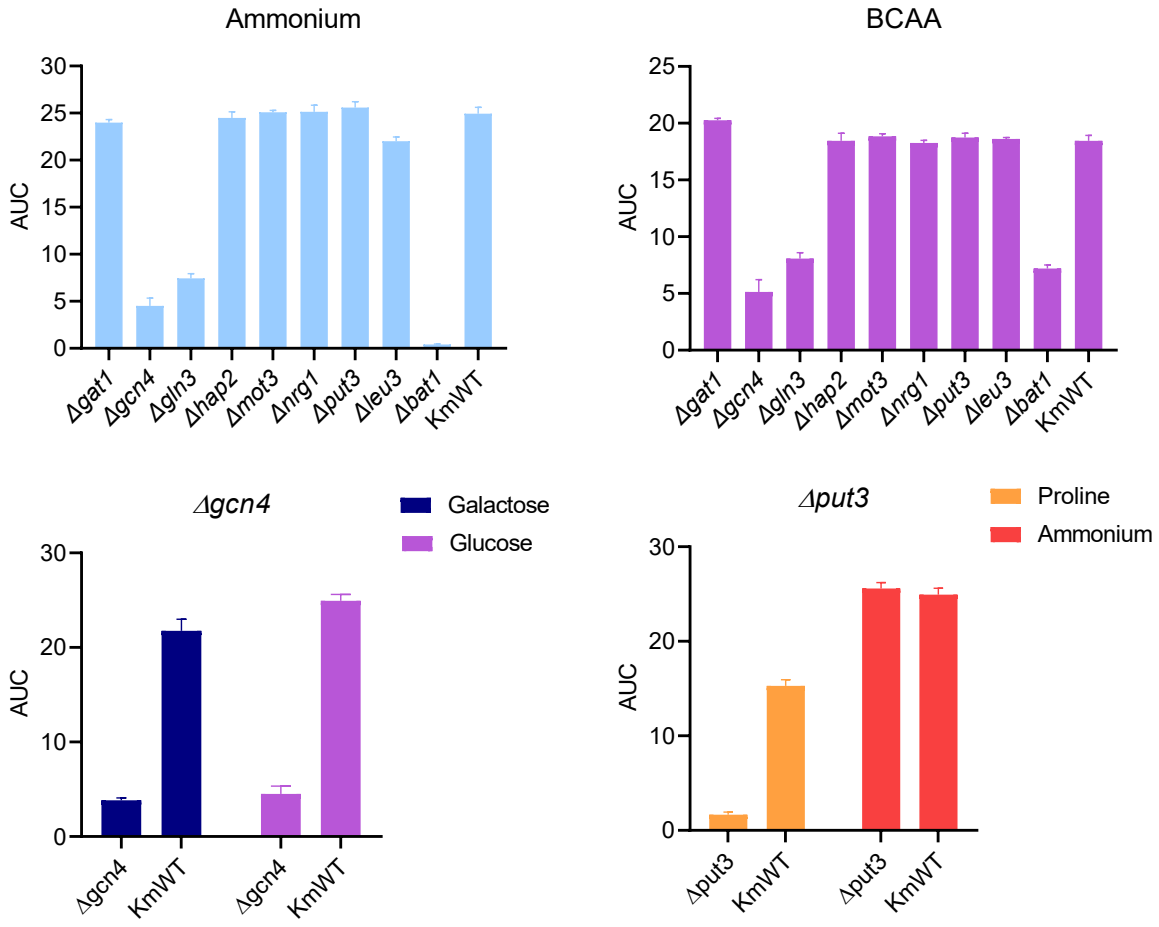
RF-dBAT1-R	GTTATGTATGTACTTGGCTGACTAGCTCAGTTCAAATCAGCAACAATACGGGACCAACCTTTGGCCCTTG	
Diag-dBAT1-F	TTGGTGAAAAGTTTTAAAGGTTTAATCGTGAACT	
Diag-dBAT1-R	TATACAGATAATGATAATATCGTTATGTATGTACTTGGCTGAC	
BSA-R	TACACGCGTTTGTACAGAAAAAAAAGAAAAATTTGA	General
K1F	TTTGCTGGCCTTTTGCTC	
K20R	TATTCTGGGCCTCCATGTC	

Supplementary material. Describing the construction of the *K. marxianus* transcription factor mutants

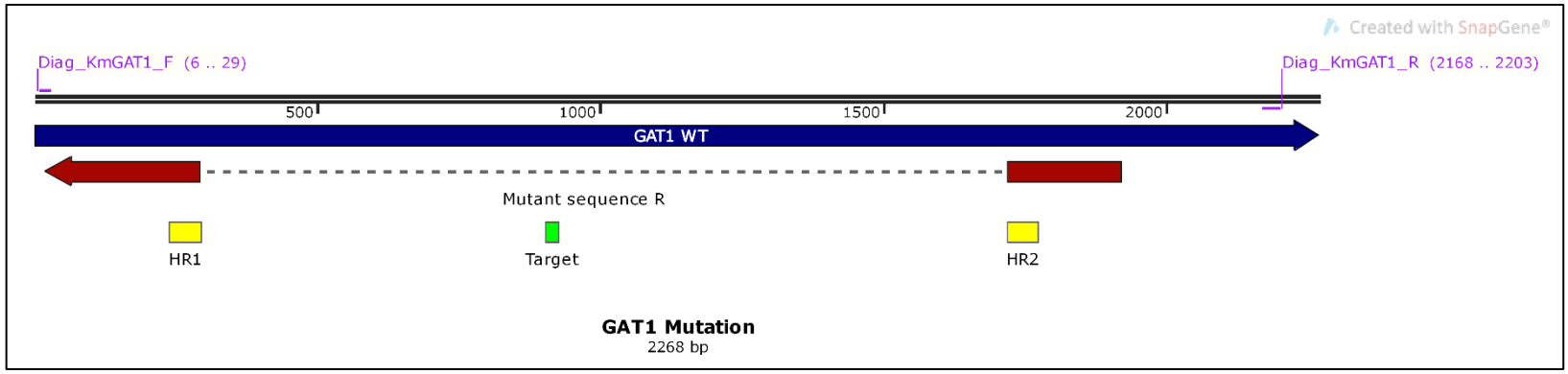
Mutants genotype and phenotype:

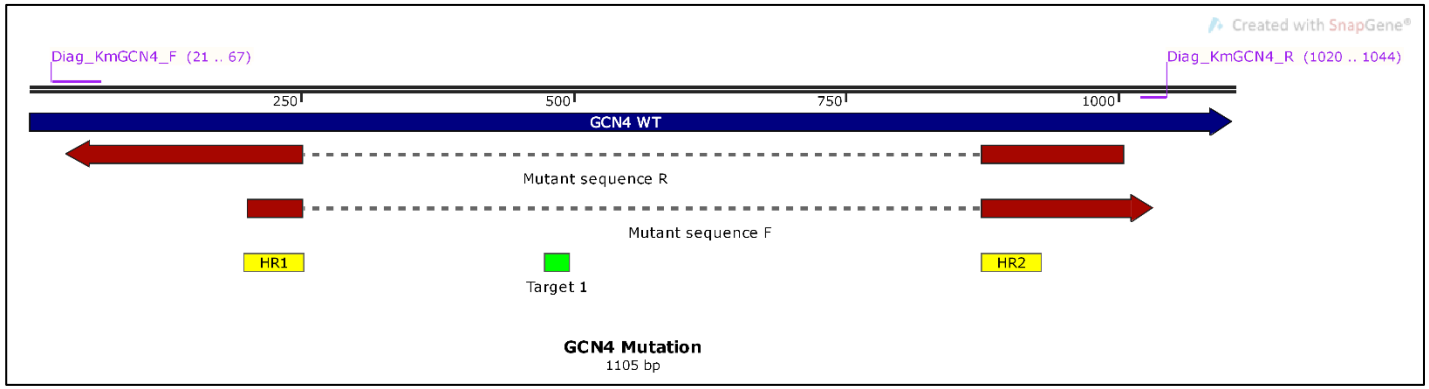
Strain name	Genotype of mutants	Deleted sequence (bp)	Growth phenotype			
			Ammonium	BCAA	Proline	Galactose
K. marxianus NBRC1777 *Δgat1*	CHR VI Δ635,534 – 636,959	1426	Good	Good	-	-
K. marxianus NBRC1777 *Δgcn4*	CHR V Δ868,076 – 868,697	622	Poor	Poor	-	Poor
K. marxianus NBRC1777 *Δgln3*	CHR III Δ1,456,475 – 1,457,877	1403	Poor	Poor	-	-
K. marxianus NBRC1777 *Δhap2*	CHR III Δ1,550,967 – 1,551,414	447	Good	Good	-	-
K. marxianus NBRC1777 *Δmot3*	CHR VI Δ700,804 – 701,788	984	Good	Good	-	-
K. marxianus NBRC1777 *Δnrg1*	CHR IV Δ283,460 – 284,113	653	Good	Good	-	-
K. marxianus NBRC1777 *Δput3*	CHR VI Δ343,875 – 345,730	1854	Good	Good	Null	-
K. marxianus NBRC1777 *Δleu3*	CHR VI Δ946,030 – 947,790	1760	Good	Good	-	-
K. marxianus NBRC1777 *Δbat1*	CHR I Δ1,068,093 – 1,069,223	1130	Null	Poor	-	-
K. marxianus NBRC1777 *ΔNTE-BAT1*	CHR I Δ1,068,052 – 1,068,127	75	Good	Good	-	-
K. marxianus NBRC1777 WT	-	-	Good	Good	Good	Good

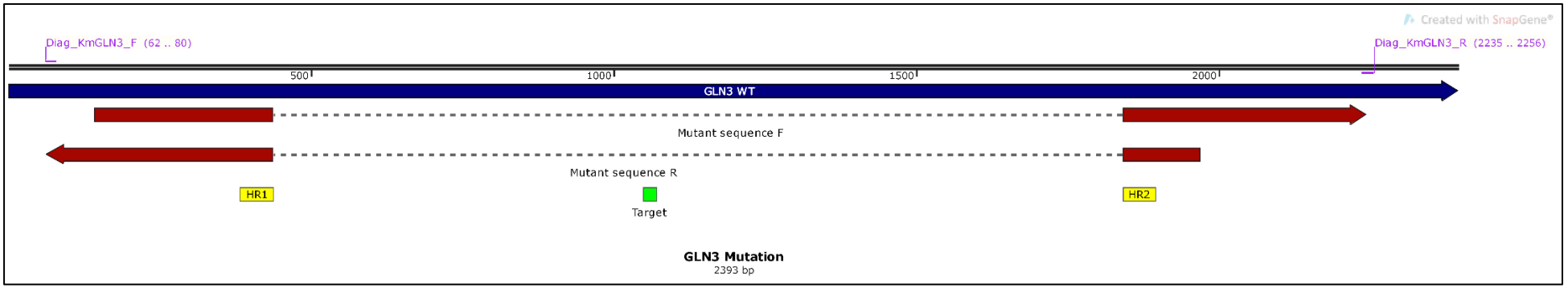
Growth of mutants in different conditions compared to the wild type:

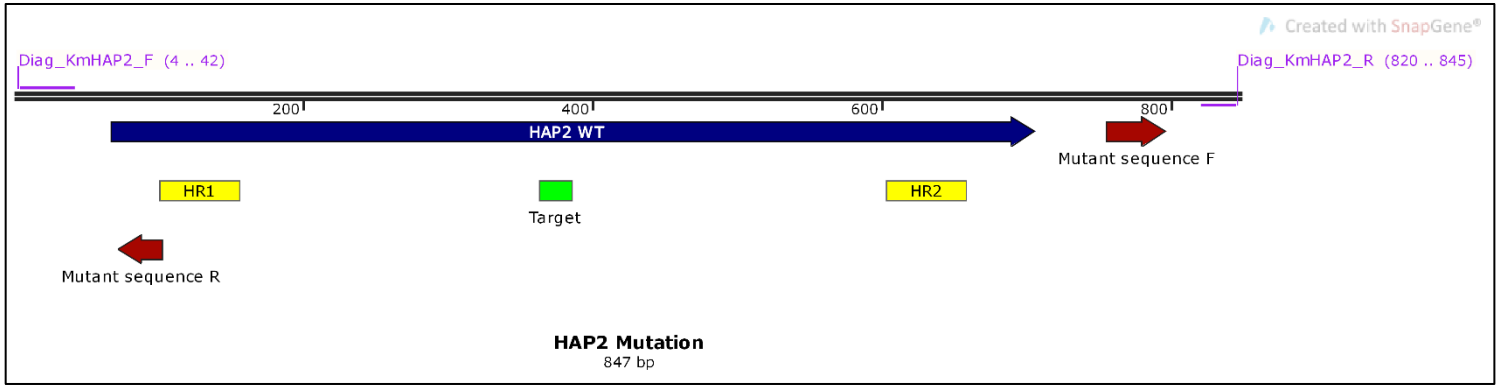


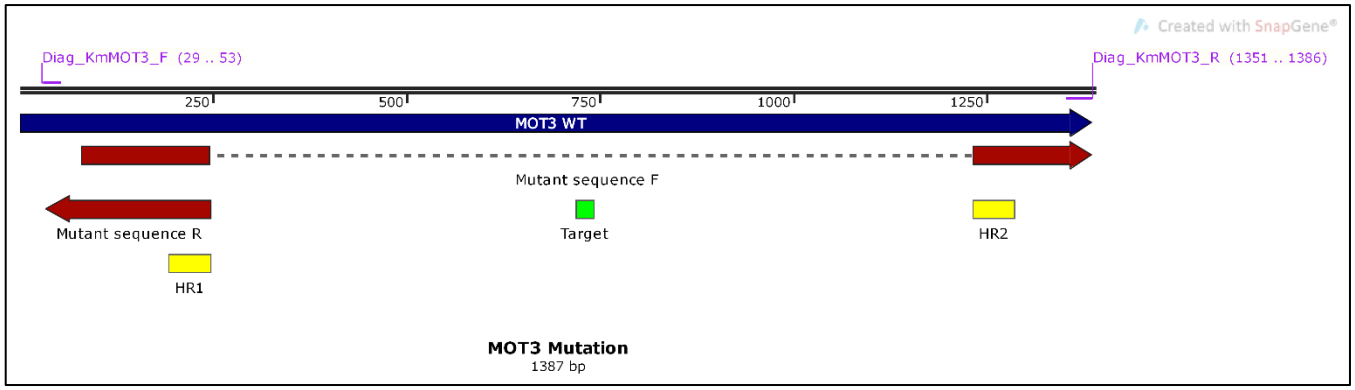
Deletions in the target locus:

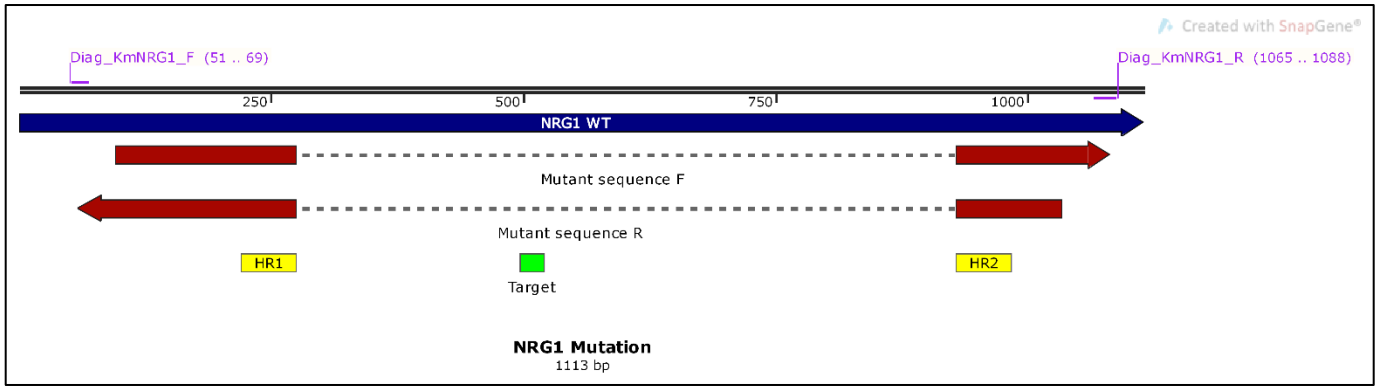


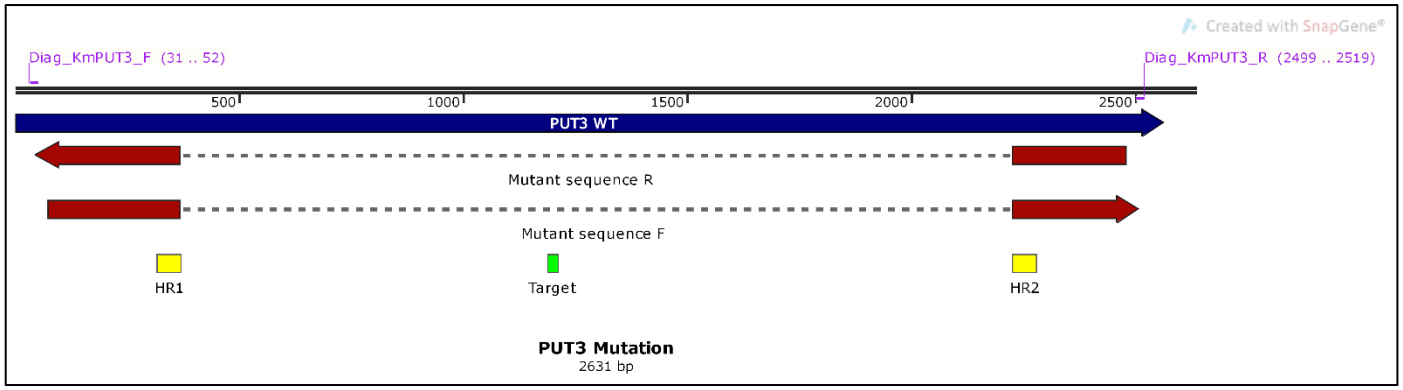


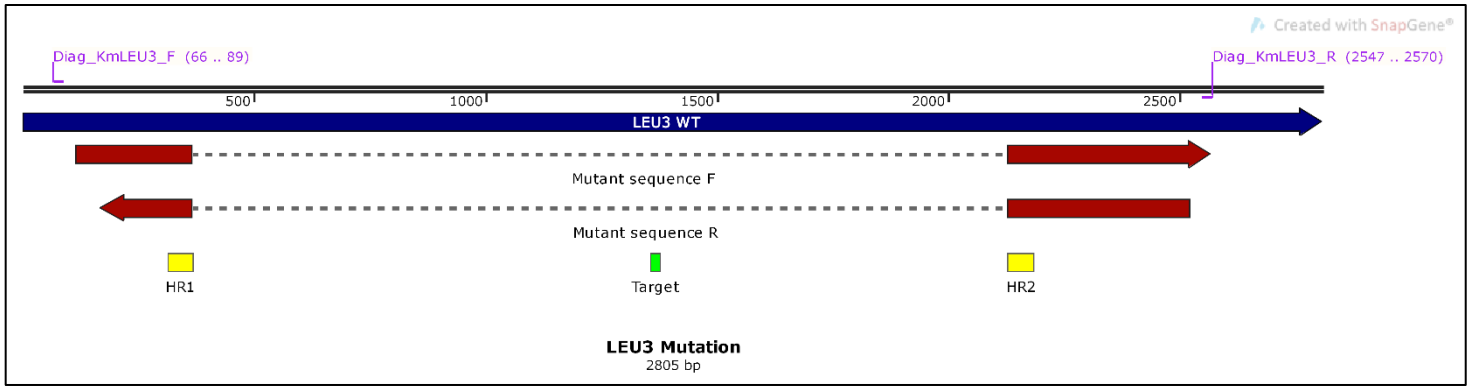

Created with SnapGene®
Diag_KmGLN3_F (62 .. 80)
Diag_KmGLN3_R (2235 .. 2256)
500
1000
1500
2000
GLN3 WT
Mutant sequence F
Mutant sequence R
HR1
Target
HR2
GLN3 Mutation
2393 bp

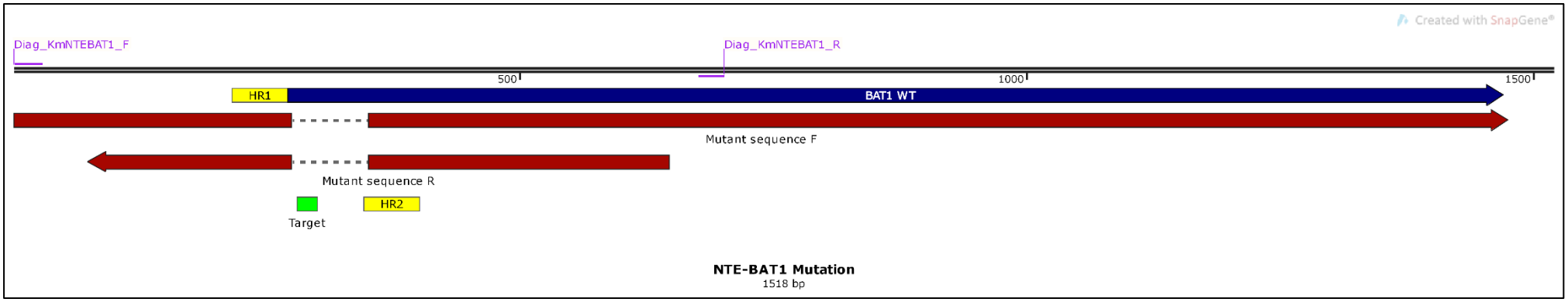

Created with SnapGene®
Diag_KmHAP2_F (4 .. 42)
Diag_KmHAP2_R (820 .. 845)
200
400
600
800
HAP2 WT
Mutant sequence F
HR1
Target
HR2
Mutant sequence R
HAP2 Mutation
847 bp

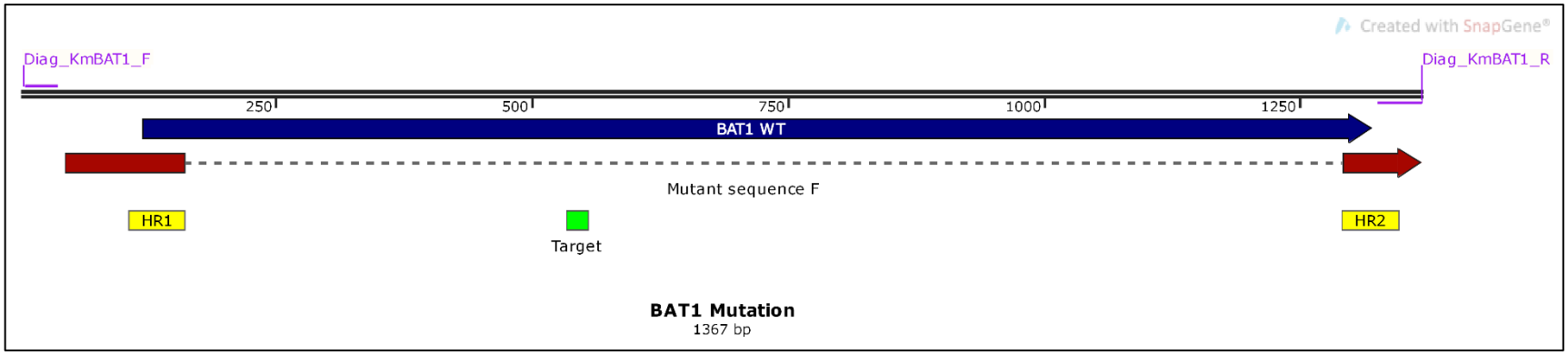

Created with SnapGene®
Diag_KmMOT3_F (29 .. 53)
Diag_KmMOT3_R (1351 .. 1386)
250
500
750
1000
1250
MOT3 WT
Mutant sequence F
Mutant sequence R
Target
HR2
HR1
MOT3 Mutation
1387 bp


Created with SnapGene®
Diag_KmNRG1_F (51 .. 69)
Diag_KmNRG1_R (1065 .. 1088)
250
500
750
1000
NRG1 WT
Mutant sequence F
Mutant sequence R
HR1
Target
HR2
NRG1 Mutation
1113 bp


Created with SnapGene®
Diag_KmPUT3_F (31 .. 52)
Diag_KmPUT3_R (2499 .. 2519)
500
1000
1500
2000
2500
PUT3 WT
Mutant sequence R
Mutant sequence F
HR1
Target
HR2
PUT3 Mutation
2631 bp

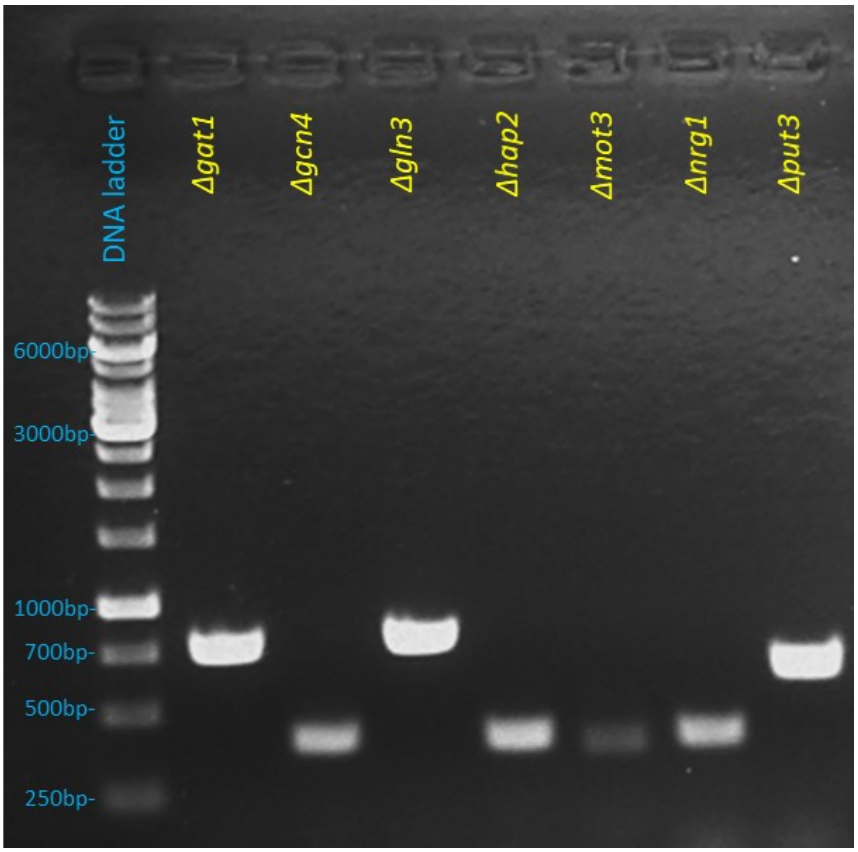

Created with SnapGene®
Diag_KmLEU3_F (66 .. 89)
Diag_KmLEU3_R (2547 .. 2570)
500
1000
1500
2000
2500
LEU3 WT
Mutant sequence F
Mutant sequence R
HR1
Target
HR2
LEU3 Mutation
2805 bp

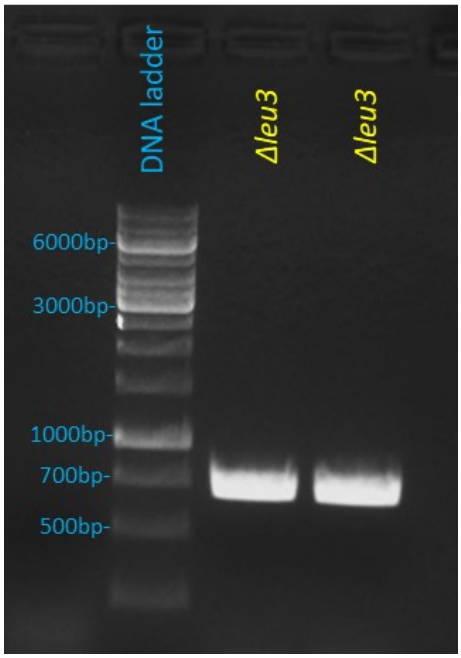

Created with SnapGene®
Diag_KmNTEBAT1_F
Diag_KmNTEBAT1_R
500
1000
1500
HR1
BAT1 WT
Mutant sequence F
Mutant sequence R
HR2
Target
NTE-BAT1 Mutation
1518 bp

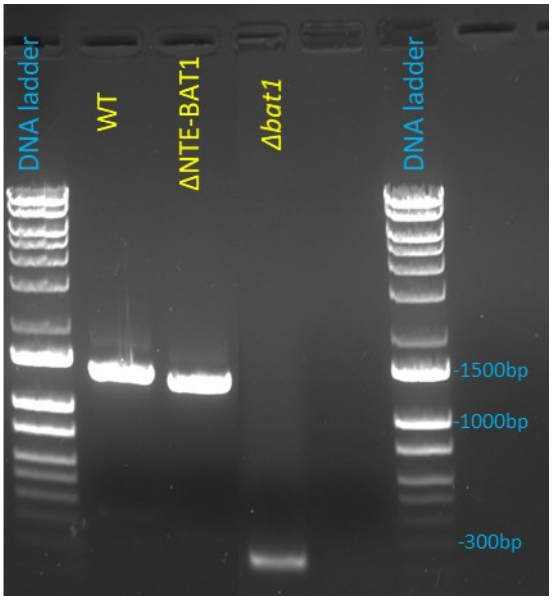

Created with SnapGene®
Diag_KmBAT1_F
Diag_KmBAT1_R
250
500
750
1000
1250
BAT1 WT
Mutant sequence F
HR1
HR2
Target
BAT1 Mutation
1367 bp

Gel electrophoresis of deletions:

Strain name	Primers	Wild type fragment length (bp)	Mutant fragment length (bp)
K. marxianus NBRC1777 *Δgat1*	Diag-dGAT1-F Diag-dGAT1-R	2198	772
K. marxianus NBRC1777 *Δgcn4*	Diag-dGCN4-F Diag-dGCN4-R	1024	402
K. marxianus NBRC1777 *Δgln3*	Diag-dGLN3-F Diag-dGLN3-R	2195	792
K. marxianus NBRC1777 *Δhap2*	Diag-dHAP2-F Diag-dHAP2-R	842	395
K. marxianus NBRC1777 *Δmot3*	Diag-dMOT3-F Diag-dMOT3-R	1358	374
K. marxianus NBRC1777 *Δnrg1*	Diag-dNRG1-F Diag-dNRG1-R	1038	385
K. marxianus NBRC1777 *Δput3*	Diag-dPUT3-F Diag-dPUT3-R	2489	635
K. marxianus NBRC1777 *Δleu3*	Diag-dLEU3-F Diag-dLEU3-R	2505	745
K. marxianus NBRC1777 *Δbat1*	Diag-dBAT1-F Diag-dBAT1-R	1350	220
K. marxianus NBRC1777 *ΔNTE-BAT1*	Diag-dBAT1-F Diag-dBAT1-R	1350	1275
K. marxianus NBRC1777	Diag-dBAT1-F Diag-dBAT1-R	1350	-




DNA ladder
Δleu3
Δleu3
6000bp-
3000bp-
1000bp-
700bp-
500bp-


DNA ladder
WT
ΔNTE-BAT1
Δbat1
DNA ladder
-1500bp
-1000bp
-300bp

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Conclusion

In the following conclusion section, the main areas covered in the thesis and the key research findings will be first summarized in relation to the research questions, reflecting on the research objectives, and describing the major contributions and applications in terms of theory and practice. This will be put in context of the challenges and limitation of studies that move beyond straightforward model systems to those that attempt to combine fundamental knowledge generation with biotechnological applicability. Finally, future perspectives and opportunities are discussed.

This study focused on non-traditional yeast and set out to investigate how the genotype, pattern of gene expression and metabolic pathways interact with the environment, with special focus on the nitrogen source as an environmental condition of interest. Our results presented in chapter 1 demonstrated that there are significant differences between *S. uvarum* and *S. cerevisiae* regarding nitrogen assimilation that are manifested at the phenotypic and metabolic levels. Further findings showed that in oenological conditions, there is a unique distribution of carbon flux in the metabolic network of *S. uvarum* to fulfil anabolic requirements and maintain redox balance. These particularities of *S. uvarum* are intensified depending on the nitrogen source, associated with the efficiency of amino acids and ammonium in supporting growth and fermentation.

In chapter 2, the nitrogen metabolism in *S. uvarum* and the influence on the production of aroma volatiles was explored. For this, transcriptomic and metabolomic analysis were done to find the association of expressed genes and the aroma metabolites produced. The data indicated that although the nitrogen regulation mechanisms of *S. uvarum* are similar to what has been reported in *S. cerevisiae*, the transcriptional response of *S. uvarum* to Ehrlich amino acids activates some aroma related genes that might have an important effect on the production of aroma volatiles. Moreover, the

investigation showed that the production of flavour and aroma compounds is more related to the nature of the nitrogen source and active catabolic routes than the growth rate sustained by the nitrogen source. This is essential knowledge to exploit the potential of *S. uvarum* in the food and beverage sectors. The understanding of the yeast genetic response to these environmental factors linked to flavour and aroma offers potential to develop novel fermented or distilled beverages with special characteristics.

This work contributed to existing knowledge in two main areas: the winemaker's innovation sector and yeast metabolism research. This study on *S. uvarum* showed that the type of nitrogen source influences yeast growth, sugar consumption and aroma profile. Although in the food and beverage sector the use of GMO is restricted, the use of genetic techniques is helpful to study the yeast genetic response as the phenotype is altered by the gene expression. The knowledge contribution of this thesis may be applied in the fermented beverage industry by using yeast strains that carry certain genes. Given the ease with which *S. uvarum* hybridises, there is the potential to use non-GM methods to introduce selected genes into host strains. The use of the appropriate strain together with a suitable formula of nitrogen content can greatly impact the quality of the fermented beverage, especially the organoleptic profile. Other intrinsic parameters of the fermentation process, such as yeast growth and fermentation kinetics, can also be optimized by the means of strain and nitrogen selection. Nevertheless, it is worth mentioning that innovation in the wine sector is restricted due to reliance on traditional practices and consumer preferences for organic and traditional approaches to wine production. Furthermore, often winemakers are reluctant to reveal precise practices that may be proprietary or advantageous, thereby liming scope for knowledge-led development and improvement. It can be a challenge for wine researchers to convince practitioners that innovation brings a significant value to the wine industry.

The work reported in chapters 3 and 4 of this thesis intended to determine the function, expression, evolution and regulation of specific genes involved in nitrogen and aroma production pathways (*ASP3* and *BAT1*). One specific purpose was to explore the evolutionary history of *ASP3* in *S. cerevisiae* and address the potential physiological role of *ASP3* in fitness of *Saccharomyces* yeasts. The investigation showed that *ASP3* is ancestral in *S. cerevisiae,* and that the gene has been lost multiple times through evolution. Our data revealed some interesting apparent contradictions. While *ASP3* is often absent, it is enriched in strains that are associated with beverage fermentations, suggesting a benefit. On the other hand, we experimentally demonstrated that a strain carrying this gene presents no advantage in fermentative environments where a mix of nitrogen compounds is available. Although, a final resolution was not achieved, a credible hypothesis is that carrying *ASP3* could be beneficial in particular ecological settings where the yeast strain is interacting with other microbes in a mixed community. It would be very interesting to explore this aspect of community evolution and community ecology in the future. Focusing on microbial communities of fermentation instead of single strains would broad our understanding of the fermentation process. The approach would be to perform community metagenomics to track genetic interactions and gene roles. Likewise, metabolomics of mixed communities would allow prediction of resultant quality of a fermented product and would help selection (design) of mixed inoculants for commercial purposes instead of single cultures, that would better represent traditional fermentations naturally happening from native microbiota. The inherent variability with such a strategy at present is a risk for wine-makers but by implementing a knowledge-led approach, it could be possible to satisfy consumer demand for authentic processes as well as the producers' need for a reliable robust process. It would also be very interesting to consider what other genetic factors play a role in metabolism and metabolic networks. For example, there could be further focus on other relevant genes performing the same or similar function, such as *ASP1*, and to explore how the presence of those alternative genes influenced the

evolutionary trajectory of *ASP3*. It would also be interesting to undertake population genetics analysis to determine whether there are any particular associations, for example SNPs or haplotypes, linked to *ASP3*. In general terms, we conclude that combining bioinformatic analysis with molecular genetics and fermentation approaches is the right track to prove evolutionary hypotheses. The evolutionary aspect was also addressed in chapter 4, with a different yeast, *K. marxianus*, where *BAT1* was investigated. We found that a single gene gives rise to a mitochondrial and cytosolic proteoform, by the use of alternative TSS depending on the nitrogen conditions. This part of the study provided valuable insights into the evolution of metabolic pathway regulation. The findings suggest that gene duplication and paralog retention provide greater regulatory control in *S. cerevisiae*. The regulation of *BAT1* in different yeast species could provide a good paradigm for the study of evolution of gene regulation, being relevant to the scientific community in yeast research. The study highlighted how insights into one species (*S. cerevisiae* in this case) can be gained by studying a different species (*K. marxianus*) and can be taken as an illustration of how a holistic approach, that is not set with narrow conceptual boundaries, adds a lot of value to a study.

While the study provided a wealth of new and valuable information, it is worth considering what aspects were most relevant and how the work could be extended. One area that was extensively considered was the choice of growth medium and experimental growth conditions. There is always a decision to be made between defined and controlled conditions versus real-life scenarios and in this project, efforts were made to balance this.

First of all, there were accurate approaches used for the development of this project that can be adopted for research of yeast in fermented beverages context. The use of synthetic must, natural must and minimal media supplemented with different nitrogen compounds directs the investigation on nitrogen metabolism in traditional conditions applied in alcoholic beverages production. It was wise to test nitrogen sources as a sole and also

as a mix, as this allowed association of the yeast genotype and phenotype to a specific compound. A valuable contribution of this study was to focus on specific genes related to nitrogen and aroma compound production since the presence and regulation of single genes can have an important influence in several biological processes. We used minimal medium in cases where we wanted to perform deep analysis on yeast metabolism to understand the effect of a single nitrogen compound on yeast behaviour. The use of simple media allowed comparison with other studies, as it is broadly used for research purposes in different organisms. By these means we had tight control over quantities of each component, so the consumption by yeast could be measured in the course of fermentation and the new generated products could be easily distinguished in the media. In other cases, we used synthetic must that mimics the grape juice. In such case the medium is more complex, but it is also a controlled approach to investigate yeast in oenological conditions. It was possible to focus on the effect of the nitrogen source by replacing the nitrogen content of the reference medium with the compound of interest. For this purpose, we established a fixed nitrogen concentration and supplemented other nitrogen compounds achieving equal amount of molecular nitrogen, identical to the standard formula. In this way it was possible to have the nitrogen type as a variable and set the nitrogen concentration constant. Other parts of the investigation required the use of natural matrices (apple juice to study *ASP3* and white grape juice to study behaviour of *S. uvarum*). The challenging aspect was to sterilise the natural juices to prevent interference from other microorganisms, without altering the properties of the media. Moreover, when using natural matrices, we had to gather information of the origin, treatments and obtention practices, and also carefully evaluate the initial composition. In experiments done with natural media, the complete understanding of the fermentation process was more limited since these raw materials are much more complex, where yeast gets exposed to a number of stresses that impact the fermentation performance.

One of the challenges in this study was to mitigate the impact of growth stage variance between conditions in batch cultures. As discussed before, the nitrogen source affects growth, thus, different growth patterns were observed between cultures grown in different nitrogen compounds. The variance of growth behaviour affects gene expression and stress conditions. Considering this limitation, we correlated growth with other parameters (fermentation performance and metabolome). Notwithstanding this limitation, for the transcriptome analysis of *S. uvarum*, it was prudent to first study the fermentation progress in each batch condition based on sugar consumption, so the dataset could be compared. Nevertheless, it would be more accurate to do the same analysis from chemostat cultivation, where the specific growth rate is the same in all conditions. In such case, it is expected that some growth-related genes, such as the ribosomal genes seen in asparagine condition, would have more similar expression levels among the nitrogen conditions. However, a major drawback of chemostats is that the limitation of growth rate by a nutrient may induce other type of bias. As with some of the other aspects already discussed, now having excellent data from batch cultures, and the experience of how strains perform in simulated medium (synthetic musts with variable nitrogen), the foundation is there to perform future growth-rate limited experiments (chemostats).

It is important to recognise that the study was performed with a restricted number of strains. This was unavoidable as it was not possible to simultaneously assess a large number of experimental variables with many strains. For example, in this study we focused mainly on one strain of *S. uvarum* as a representative to enable an integrated analysis of metabolome and transcriptome analysis. This brings a certain limitation as findings with a small number of representatives may not be applicable to all strains of a species. This is always the case, however, even with *S. cerevisiae* where population genomics has revealed tremendous diversity even amongst commercial strains. This is likely to be even more relevant to *S. uvarum* where the influence of domestication is weaker and thus the intrinsic

diversity is large. Nonetheless, in spite of that limitation, this study has shown how particular genetic traits considerably affect the fermentation profile. There is great scope to expand this analysis to larger populations where we now have a narrower set of variables, thereby allowing us to increase the sample size. In this regard, we also have to recognise that there are limited genomic data available for non-traditional yeasts. Most of the genome sequences of our strains of interest have poor quality, making it hard to have robust bioinformatic data. Specially in subjects such as the transcriptomic analysis of *S. uvarum*, the synteny analysis of *ASP3* in *S. cerevisiae* and the transcriptional regulation of *BAT1* in *K. marxianus*, we were limited in this regard. Moreover, the genetic methodologies are standardized mostly in the conventional yeast *S. cerevisiae*, which first, entails methods optimization when switching host, second, impedes rapid investigations, and third, creates bias when comparing results to non-conventional yeasts. These facts challenge yeast researchers to keep exploring non-conventional yeasts and generate genomic information at intraspecies level. One aspect where there is likely to be huge progress in the future is in the use of genetic technologies to identify traits of interest and improve strains. Taking *S. cerevisiae* as a reference, there are many examples of where techniques like QTL mapping and adaptive laboratory evolution (ALE) have been applied to improve strains for application in biotechnology. These could also be applied now in *S. uvarum,* for example.

In addition to exploiting the diversity within a non-traditional species like *S. uvarum*, future studies could go further in comparing genetic sequence and kinetics of specific enzymes related to aroma in other, more divergent non-traditional yeasts. We started in this direction with *K. marxianus* where we could see more profound differences in transcriptional regulation mechanisms between *S. cerevisiae* and *K. marxianus* than would be seen within the *Saccharomyces* genus. Consequently, a future focus on the characteristics of specific aroma and nitrogen related proteins will be important to explain the differences between yeast species.

There were a number of very specific areas where future work could progress from findings of this thesis.

- *YJL213W* is an example of the knowledge gap existing regarding proteins related to nitrogen utilization in yeast, from which several genes of unknown function remain to be investigated. Future research might explore proteins with important role in the fermentation profile in non-traditional yeast, and relay on heterologous expression of the orthologous proteins in *S. cerevisiae* to understand the effect on a fermentation context.
- The next step as continuation of this work in *S. uvarum*, would be to evaluate growth, metabolome and transcriptome under different temperatures to determine whether this factor has a role in aroma metabolism, relaying on comparison with *S. cerevisiae.* This work in oenological conditions was done at 20°C since it is low temperature for wine fermentation, however, cider fermentations where *S. uvarum* is commonly used, are often fermented at 8°C - 14°C, so clearly, this yeast has a good potential to ferment at lower temperatures that would be interesting to explore further.
- The study of *ASP3* is a fruitful area for further work. We identified great research potential on studying the role of yeast communities naturally found in artisanal (craft) cider fermentation, exploring microbial interactions at chemical and genetic level. From that pool of yeast species, we can estimate the common up-regulated genes that might have an important role in fermentation and aroma profile. This will elucidate gene sharing mechanisms and will clarify the importance of *ASP3* in fermentations with natural matrices.
- Finally, in regard to *BAT1* transcriptional regulation in *K. marxianus*, there are interesting questions that could be further explored. Some aspects of the regulatory system were uncovered but the interaction between multiple transcription factors and the possible role of chromatin structure in selection of alternative TSS needs to be

addressed. It will also be curious to determine the extent to which this particular mechanism is found for other genes encoding proteins with more than one sub-cellular location, in *K. marxianus* and in the orthologous genes in other yeasts. That latter inter-species comparison can shed further light on the evolutionary steps and processes that occurred in the progression from the *K. marxianus*-like single gene system to the duplicated genes in *S. cerevisiae*. There are also questions that apply to both systems – for example, although there is regulation of synthesis of the cytosolic and mitochondrial forms, it is still the case that cells appear to survive with a single form – suggesting that the sub-cellular localisation is not vital. This questions why elaborate regulatory mechanisms that appear not to be essential evolve? The answer to that may be that the conditions that drove the evolution, and indeed the conditions where the regulation is important, are not sufficiently understood. These points are both of academic interest and relevant for application given the critical role of amino acid metabolism is fermentation and in aroma formation.

The work reported in this thesis has made substantial contributions to knowledge on the role of non-traditional yeasts on industrial fermentations, focusing on the effect that this organisms have on flavour and aroma of fermented beverage. The findings of this work raised intriguing questions regarding the nature and capacity of the nitrogen source to influence yeast metabolism. It is abundantly clear that scientific investigations on non-*Saccharomyces* yeasts are still in the early stages. Although species like *S. uvarum* and *K. marxianus* have been used in traditional beverages like cider and kefir for hundreds, if not thousands of years, modern producers still rely on craft rather than science. This thesis work can be an important step towards the integration of craft and science as we attempt to create new innovative products while respecting the legacy given us by nature.

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