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An indirect assay for volatile compound production in yeast strains

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Traditional flavor analysis relies on gas chromatography coupled to mass spectrometry (GC-MS) methods. Here we describe an indirect method coupling volatile compound formation to an *ARO9*-promoter-*LacZ* reporter gene. The resulting β-galactosidase activity correlated well with headspace solid phase micro extraction (HS/SPME) GC-MS data, particularly with respect to the formation of rose flavor. This tool enables large-scale screening of yeast strains and their progeny to identify the most flavor active strains.

The organoleptic perception of beer depends mainly on substances produced by yeast during the fermentation process. Flavor active substances are volatile compounds and include higher alcohols, esters, and fatty acids. In the wine industry attempts are made to increase flavour compounds by either simultaneous or sequential co-fermentations using either different yeast strains, i.e *Saccharomyces cerevisiae* with a non-*Saccharomyces* yeast, or mixing bacterial strains, e.g. *Oenococcus oeni*, with wine yeasts[5,8,14,17]. Research interest in natural flavors produced by yeasts has gained increasing interest, particularly focusing on isoamyl alcohol (banana flavor) and β-phenylethanol (flowery, rose flavor). Both compounds are produced during amino acid catabolism in yeast[9]. The Ehrlich pathway, a linear pathway requiring three enzymatic activities, is responsible for converting aromatic amino acids (phenylalanine, tyrosine, and tryptophan), branched-chain amino acids (leucine, isoleucine, and valine) and methionine into higher alcohols. The regulation of the Ehrlich pathway depends at least in part on the Zn_2Cys_6 transcription factor Aro80, which regulates *ARO9* and *ARO10* in a nitrogen source dependent manner (Fig. 1A)[12]. One of the key bottle necks in flavor research is the requirement of chemical analytical tools to measure volatile compounds produced during fermentation, which is generally done using HS/SPME extraction methods coupled to GC-MS[2,15]. This method, however, is time consuming, requires additional quantitation as well as prior lab scale fermentations and sample preparations, which are often difficult to optimize for high throughput screening.

In order to identify a promoter that is most responsive to *ARO80* overexpression, we co-transformed *ARO80* under the control of the *Ashbya gossypii TEF*-promoter with plasmids containing *ARO8, ARO9, ARO10*, and *ARO80* promoter-*lacZ* reporter gene fusions into *S. cerevisiae* (Fig. 1B). To investigate whether expression of the reporter genes was actually Aro80-dependent we quantified β-galactosidase activity in strains bearing the endogenous *ARO80*, an *ARO80* deletion, or the *ARO80* overexpression construct (Fig. 1C). This established the *ARO9* as a potential reporter for a strain's flavor production.

To correlate *ARO9* reporter gene activity with flavor formation we first determined its activity in a set of strains with *S. cerevisiae* background expressing *ARO80* at wild type levels. This included the laboratory strain CENPK, two hybrid lager yeast strains, collectively known as *S. pastorianus* as well as a Bordeaux wine yeast. For comparison we used these strains in bench-top fermentation assays and at the end of fermentation volatiles were extracted by HS/SPME and analyzed via GC-MS (Tab. S1). For the comparison of volatile compound formation with β-galactosidase activity we focused our attention to phenylalanine catabolites (rose flavor). This showed that β-galactosidase activity of the *ARO9-lacZ* reporter correlated well with the amount of β-phenylacetate and β-phenylethanol produced by these strains (Fig. 2).

To determine the applicability of this tool beyond *S. cerevisiae* we used the *ARO9*-reporter with strains from the *Saccharomyces sensu stricto* complex including *S. bayanus, S. cariocanus, S. eubayanus, S. kudriavzevii, S. mikatae, S. paradoxus*, and *S. uvarum* (Fig. 2). The flavor profiles show that there is a great variability in volatile formation between these strains (Tab. S1). This variability is also reflected in the β-galactosidase activity in these strains indicating that high β-galactosidase activity pairs with increased flavor production. A correlation curve was analyzed comparing β-galactosidase activity with the combined flavor values for 2-phenyl ethanol and 2-phenyl acetate (Fig. 2C). This took into account that Aro9 enzymatic activity is upstream of 2-phenyl ethanol and 2-phenyl acetate production.

Figure 1 | Identification of a reporter gene for Ehrlich Pathway activity. (A) Amino acids (branched-chain amino acids, leucine, isoleucine, and valine, aromatic amino acids, phenylalanine, tyrosine, and tryptphan, or methionine) are converted in the Ehrlich pathway to fusel alcohol or fusel acids in a three step process. The genes encoding enzymes that catalyze single steps are indicated. Oxidation of aldehydes to fusel acids is done by aldehyde dehydrogenases (e.g. *ALD1*). Reduction of aldehydes to fusel alcohols is done by alcohol dehydrogenases (e.g. *ADH1*). Transcriptional regulation by Aro80 and co-factor requirement is indicated. (B) Plasmids carrying the *ARO80* overexpression and one of the *ARO*-promoter-*lacZ* reporter gene constructs were co-transformed into *S. cerevisiae* (BY4741). (C) Quantitative β-galactosidase assay with strains bearing the indicated *ARO*-promoter-*lacZ* constructs in strains in which *ScARO80* was either overexpressed or deleted, or contained the wildtype *ARO80*.

Fermented beverages contain only small amounts of volatile compounds; yet, these are of paramount importance for the flavor profile and organoleptic perception of a beverage[19,20]. Changes in brewing technology, e.g. introduction of high-gravity brewing, can drastically alter the flavor composition - in this case - by resulting in an increase in the amount of acetate esters. Consumer preference is towards all natural flavors and unique flavor signatures[10]. Based on this non-GMO preference, three main roads are currently followed to improve flavor content of beverages: (i) choice of the starter culture, (ii) mixed fermentations using different yeast species or a combination of yeast and bacterial species, and (iii) selection of strains high in volatile compound formation via yeast breeding approaches[1,5,6,22].

For example, yeasts belonging to the genera *Hanseniaspora* and *Pichia* are good producers of acetate esters, whereas mixed fermentations with *S. cerevisiae* and *Lachancea thermotolerans* increased the level of β-phenylethanol[4,21]. Furthermore, mixed fermentations, including *S. cerevisiae* and a bacterial strain e.g. *Oenococcus oeni*, promise to provide novel flavor variations[17].

With the highly advanced gene function analyses in *S. cerevisiae* the genetic repertoire involved in volatile compound formation has been elucidated to a great extent[18]. The Ehrlich pathway plays a central role in aromatic and branched-chain amino acid catabolism resulting in the conversion of amino acids to aroma compounds[9]. Several studies have described an increase in flavor production by selecting for yeast strains resistant to fluoro-amino acids. An increased production of isoamyl alcohol, for example, can be achieved by selecting mutants resistant to trifluoroleucine[3]. In such strains a mutation of D578Y in the *LEU4* gene releases feedback inhibition and initiates increased production of leucine and its catabolites[16]. Using a genetic approach it was shown that overexpression of the alcohol acetyl transferases *ATF1* and *ATF2* substantially increased the production of isoamyl acetate[20].

The indirect assay described in this study converts Ehrlich pathway activity into a reporter gene readout that can be quantified as β-galactosidase activity. We base the tool on the *ARO9* promoter as the *ARO8* promoter was not responsive to Aro80 and has been shown to be under general control[13]. With this method we can preferably assay rose flavor. Apparently, however, this reporter is not discriminatory towards branched chain amino acids (Tab. S1).

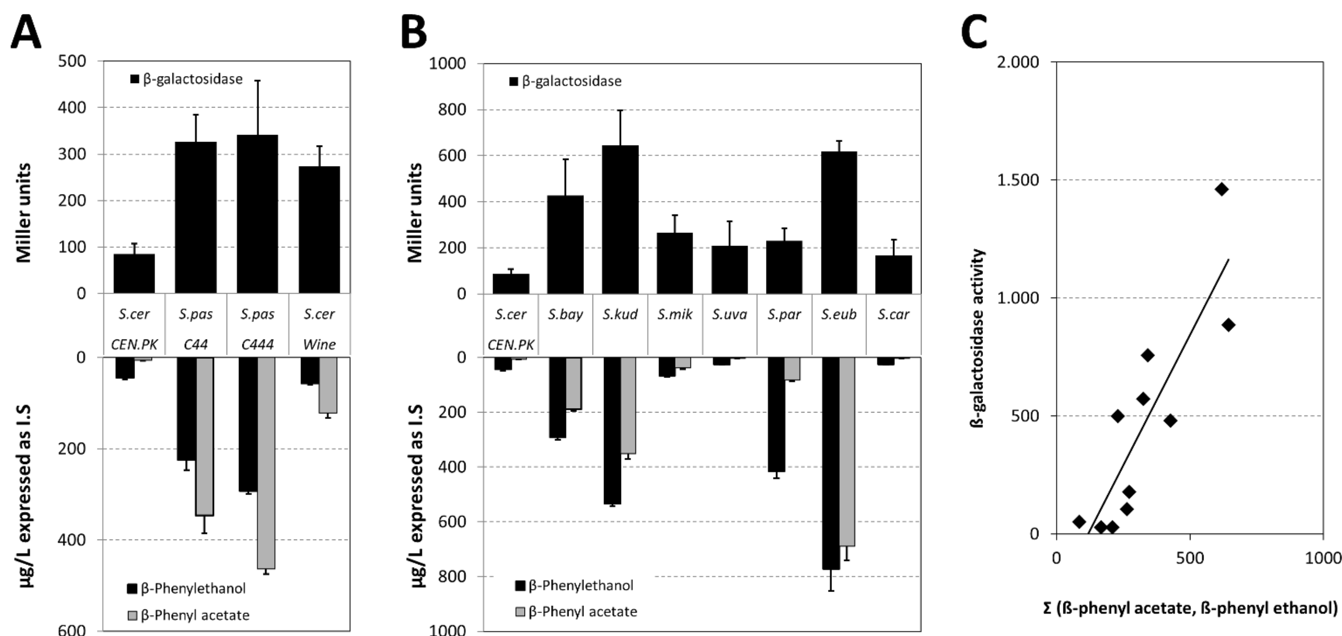

Figure 2 | Comparison of β-galactosidase activity with volatile compound formation. (A) Assay with either the indicated *S. cerevisiae* strains (A) or with *Saccharomyces sensu stricto* strains (B). Upper panels depict β-galactosidase activity based on the *ARO9p-lacZ* reporter construct. Lower panels show β-phenylethanol and β-phenylacetate volatile compounds. Note: Fermentation with the wine strain in (A), was done in YPD due to its lack of *MAL*-genes. The low amount of flavor produced by *S. mikatae*, *S. cariocanus*, and *S. cerevisiae* in (B) is due to their inability to end-ferment granulated malt used in these fermentations. Correlation of ß-galactosidase activity and the combined yield of phenylalanine catabolites are shown in (C).

Our tool is fast and convenient and can be adapted for use with high throughput microtiter plate assays in yeast[7,11]. Thus this indirect flavor assay system is inexpensive and allows screening of large libraries of yeast strains as well as F1/F2 populations of interbred strains. This will lead to the rapid identification of strains with potentially improved flavor characteristics compared to the parental strains. Additionally, different growth regimes can lead to altered flavor production. This allows the implementation of changes in oxygen supply and use of different nitrogen sources.

1. Arroyo-Lopez, F. N. *et al.* Yeasts in table olive processing: desirable or spoilage microorganisms? *Int J Food Microbiol* **160**, 42–49 (2012).
2. Canuti, V. *et al.* Headspace solid-phase microextraction-gas chromatography-mass spectrometry for profiling free volatile compounds in Cabernet Sauvignon grapes and wines. *J Chromatogr A* **1216**, 3012–3022 (2009).
3. Casalone, E. *et al.* Genetic and biochemical characterization of Saccharomyces cerevisiae mutants resistant to trifluoroleucine. *Res Microbiol* **148**, 613–623 (1997).
4. Comitini, F. *et al.* Selected non-Saccharomyces wine yeasts in controlled multistarter fermentations with Saccharomyces cerevisiae. *Food Microbiol* **28**, 873–882 (2011).
5. Domizio, P. *et al.* Outlining a future for non-Saccharomyces yeasts: selection of putative spoilage wine strains to be used in association with Saccharomyces cerevisiae for grape juice fermentation. *Int J Food Microbiol* **147**, 170–180 (2011).
6. Garcia Sanchez, R., Solodovnikova, N. & Wendland, J. Breeding of lager yeast with Saccharomyces cerevisiae improves stress resistance and fermentation performance. *Yeast* **29**, 343–355 (2012).
7. Gietz, R. D. & Schiestl, R. H. Microtiter plate transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc* **2**, 5–8 (2007).
8. Gobbi, M. *et al.* Lachancea thermotolerans and Saccharomyces cerevisiae in simultaneous and sequential co-fermentation: a strategy to enhance acidity and improve the overall quality of wine. *Food Microbiol* **33**, 271–281 (2013).
9. Hazelwood, L. A., Daran, J. M., van Maris, A. J., Pronk, J. T. & Dickinson, J. R. The Ehrlich pathway for fusel alcohol production: a century of research on Saccharomyces cerevisiae metabolism. *Appl Environ Microbiol* **74**, 2259–2266 (2008).
10. Hugenholtz, J. Traditional biotechnology for new foods and beverages. *Curr Opin Biotechnol* **24**, 155–159 (2013).
11. Ichikawa, K. & Eki, T. A novel yeast-based reporter assay system for the sensitive detection of genotoxic agents mediated by a DNA damage-inducible LexA-GAL4 protein. *J Biochem* **139**, 105–112 (2006).
12. Iraqui, I., Vissers, S., Andre, B. & Urrestarazu, A. Transcriptional induction by aromatic amino acids in Saccharomyces cerevisiae. *Mol Cell Biol* **19**, 3360–3371 (1999).
13. Iraqui, I., Vissers, S., Cartiaux, M. & Urrestarazu, A. Characterisation of Saccharomyces cerevisiae ARO8 and ARO9 genes encoding aromatic aminotransferases I and II reveals a new aminotransferase subfamily. *Mol Gen Genet* **257**, 238–248 (1998).
14. Kim, D. H., Hong, Y. A. & Park, H. D. Co-fermentation of grape must by Issatchenkia orientalis and Saccharomyces cerevisiae reduces the malic acid content in wine. *Biotechnol Lett* **30**, 1633–1638 (2008).
15. Mauriello, G., Capece, A., D'Auria, M., Garde-Cerdan, T. & Romano, P. SPME-GC method as a tool to differentiate VOC profiles in Saccharomyces cerevisiae wine yeasts. *Food Microbiol* **26**, 246–252 (2009).
16. Oba, T., Nomiyama, S., Hirakawa, H., Tashiro, K. & Kuhara, S. Asp578 in LEU4p is one of the key residues for leucine feedback inhibition release in sake yeast. *Biosci Biotechnol Biochem* **69**, 1270–1273 (2005).
17. Rossouw, D., Du Toit, M. & Bauer, F. F. The impact of co-inoculation with Oenococcus oeni on the trancriptome of Saccharomyces cerevisiae and on the flavour-active metabolite profiles during fermentation in synthetic must. *Food Microbiol* **29**, 121–131 (2012).
18. Styger, G., Jacobson, D., Prior, B. A. & Bauer, F. F. Genetic analysis of the metabolic pathways responsible for aroma metabolite production by Saccharomyces cerevisiae. *Appl Microbiol Biotechnol* **97**, 4429–4442 (2013).
19. Verstrepen, K. J. *et al.* Flavor-active esters: adding fruitiness to beer. *J Biosci Bioeng* **96** (2003).
20. Verstrepen, K. J. *et al.* Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. *Appl Environ Microbiol* **69**, 5228–5237 (2003).
21. Viana, F., Gil, J. V., Genoves, S., Valles, S. & Manzanares, P. Rational selection of non-Saccharomyces wine yeasts for mixed starters based on ester formation and enological traits. *Food Microbiol* **25**, 778–785 (2008).
22. Zott, K. *et al.* The grape must non-Saccharomyces microbial community: impact on volatile thiol release. *Int J Food Microbiol* **151**, 210–215 (2011).

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Author contributions

D.R. carried out the molecular experiments; D.R. and K.T. carried out flavor measurements; A.W., J.W.W. and U.V. designed the experiments, A.W. and D.R. prepared the figures, J.W.W. wrote the main manuscript text; all authors reviewed the manuscript.

Additional information

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