An indirect assay for volatile compound production in yeast strains

Davide Ravasio¹, Andrea Walther¹, Kajetan Tröst², Urska Vrhovsek² & Jürgen Wendland¹

¹Carlsberg Laboratory; Yeast Genetics Gamle Carlsberg Væj 10 DK-1799 Copenhagen V, Denmark, ²Fondazione Edmund Mach Research and Innovation Centre Food Quality and Nutrition Department Via E.Mach 1, I-38010 S.Michele all’Adige, Italy.

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-λacZ 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. Research interest in natural flavors produced by yeasts has gained increasing interest, particularly focusing on isoamyl alcohol (banana flavor) and β-phenylethanol (florery, rose flavor). Both compounds are produced during amino acid catabolism in yeast. 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 Zn2Cys6 transcription factor Aro80, which regulates ARO9 and ARO10 in a nitrogen source dependent manner (Fig. 1A). 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. 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-λacZ 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-λacZ 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. cubayanus, 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.
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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{1,21}. Furthermore, mixed fermentations, including S. cerevisiae and a bacterial strain e.g. Oenococcus oeni, promise to provide novel flavor variations\textsuperscript{19}.

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\textsuperscript{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\textsuperscript{5}. 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\textsuperscript{3}. In such strains a mutation of D578Y in the LEU4 gene releases feedback inhibition and initiates increased production of leucine and its catabolites\textsuperscript{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\textsuperscript{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\textsuperscript{13}. With this method we can preferably assay rose flavor. Apparently, however, this reporter is not discriminatory towards branched chain amino acids (Tab. S1).
Our tool is fast and convenient and can be adapted for use with high throughput microtiter plate assays in yeast. 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.

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 β-galactosidase activity and the combined yield of phenylalanine catabolites are shown in (C).

Acknowledgments
This research was supported in part by the European Union Marie Curie Initial Training Network Cornucopia (http://www.yeast-cornucopia.se/).

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
Supplementary information accompanies this paper at http://www.nature.com/
scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Ravasio, D., Walther, A., Trost, K., Vrhovsek, U. & Wendland, J. An
indirect assay for volatile compound production in yeast strains. Sci. Rep. 4, 3707;
DOI:10.1038/srep03707 (2014).

This work is licensed under a Creative Commons Attribution-
NonCommercial-NoDerivs 3.0 Unported license. To view a copy of this license,
visit http://creativecommons.org/licenses/by-nc-nd/3.0