Revised design of rose-scented *Saccharomyces cerevisiae*; a yeast to enhance the aroma of baked goods

Julia Ashley, Ed He, Jordan Kramer, Noah Luch, Vivien Marmerstein, Lydia Park, Alex Tang, Joy Tian, Rain Wu

BioBuilderClub, Western Reserve Academy, Hudson, Ohio, USA

Reviewed on 2 May 2020; Accepted on 22 June 2020; Published on 26 October 2020

Chefs around the world often use roses in dessert-making, including baked goods such as rose crepe cake, rose mousse, and rose pound cake. Therefore, a transformed yeast could aid in the baking process by producing rose flavor and scent during fermentation to enhance the aroma and taste of baked goods. Two enzymes—known as ScOYE and ScGES—are responsible for converting glucose to citronellol, the compound that accounts for the scent found in rose oils. Our design process includes inserting the genes that produce these enzymes into a yeast plasmid, pGDP2. After using PCR to amplify the genes, either a Golden Gate or Gibson assembly process would be used to clone the genes into the plasmid. Expression of the assembled sequence in baker's yeast will confer the characteristic rose aroma into the yeast. Future data and testing will determine how long the rose aroma remains in the yeast for extended periods of time, including the fermentation process, to produce rose-scented baked goods. A synthetically engineered yeast would allow for easier production of rose-scented goods, and the protocols derived and perfected in the process will potentially revolutionize the baking industry as it would allow for accessibility of a variety of flavorful baked goods.

Keywords: Rose, *Saccharomyces cerevisiae*, citronellol, baking

Mentors: Beth Pethel nad Irene Reizman

Direct correspondence to pethelb@wra.net

Background

Many chefs incorporate various flavors when making baked goods with baker's yeast. Rose is commonly regarded for its sweet smell and affiliation with romance, and can be used in a variety of baked goods, including rose crepe cake, rose mousse, and rose pound cake. When producing rose-infused goods, chefs use dried rose petals or some other rose substitute, which may be poses as an inconvenience due to time constraint, cost, and effective translation of flavor (Great British Chefs 2020). Authentic rose oil can cost up to $800 US dollars per fluid ounce, and utilizing dried rose petals in baking requires the extended growing/cultivating time and measures, making rose oil and crushed rose petals difficult conduits of flavor in rose baked goods. Additionally, roses are grown seasonally and take six to eight weeks to bud after planting, and are therefore

not available for cultivation at all times during the year (Bender 2020). Citronellol and geraniol are the two active components of the scent associated with roses, and this research aims to produce both through a glucose pathway (Katsukawa, Nakata, Koeji, et al. 2011). This work proposes a faster way to infuse baked goods with a rose aroma and flavor by utilizing synthetically engineered *Saccharomyces cerevisiae* to produce geraniol and citronellol. Preliminary work shows various yeast strains producing flavonoids, a type of natural product occurring in plants. In this particular experiment, six flavonoids, targeting health-related compounds, were successfully synthesized in yeast (Rodriguez, Strucko, Stahlhut, et al. 2017). Although the transformed yeasts were not intended for market, this work showed the potential for flavor and scent-producing yeast. We plan to use the ScOYE and ScGES genes to enable the chemical pathway, converting glucose, to geraniol, to citronellol, the desired final olfactory product.

S. cerevisiae, or baker's yeast, is a species of yeast most commonly used for bread making. Synthetically engineered *S. cerevisiae* have potential to improve the mass production of rose baked goods to overcome current limitations arising with procuring supplemental rose flavor for desserts. The production of a yeast that can give bread a rose odor during fermentation could enable commercialization for rose flavored baked goods, from industrial baking to the at-home chef. The proposed transformation of this yeast will bring flavoured yeast to a wide range of users.

Systems level

We plan to implement our project in six distinct steps (Figure 1). The enzymes geraniol synthase (GES) and the Old Yellow Enzyme (OYE) use the mevalonate and 1-deoxylulose 5-phosphate pathways (MVA and DXP),

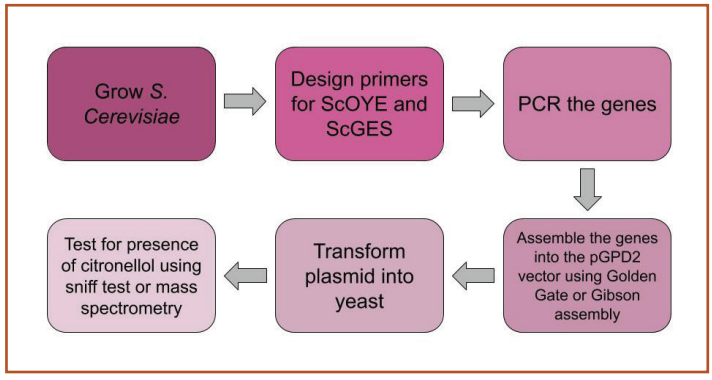

Figure 1. *This shows a simplified diagram of our planned systems level. Further detail into the processes are described below.*

two essential metabolic pathways, to convert glucose to citronellol, the desired final product. GES originates from ObGES, found in *Ocimum basilicum* or sweet basil, and OYE comes from HbOYE, which is found in Hevea brasiliensis or the Para rubber tree. Therefore, we must codon optimize the genes for *S. cerevisiae*, as indicated by the added prefix "Sc." Codon optimization is important as the genes come from organisms other than baker's yeast, so the codon expression and function will likely vary. We then plan to use standard molecular biology techniques—PCR and either Golden Gate or Gibson assembly—to amplify and ligate the ScOYE and ScGES genes into the pGPD2 vector at the location of the GAP promoter, as seen in Figure 2. The GAP promoter was chosen as its function is not inhibited by glucose, as many other yeast promoters are. As glucose is needed in the preliminary step of the pathway to citronellol, a promoter inhibited by it would be problematic. The pGPD2 plasmid was chosen as it can be transformed and replicated in both yeast and bacteria, which would be useful depending on which organism we transform with the plasmid. In addition, it already possesses the GAP promoter, making it an optimal vector for this work (Crook, Freeman and Alper 2011). The cloning will be done using a Golden Gate or Gibson assembly process.

Before transforming the cloned pGPD2 vector into *S. cerevisiae*, we would make chemically competent yeast cells. After confirming the appropriate cloning of the genes into the pGDP2 plasmid and making the competent cells by using PCR to amplify the genes with two primers, we will transform the plasmid using the Frozen-EZ Yeast Transformation II kit (Zymo Research 2020). Following this, we will select for positive clones with selective media. The chosen plasmid, pGDP2, possesses resistance to URA as indicated by the URA3 gene, so agar media with URA will be used to select for successful transformants. After growing the yeast on the selective media to isolate the organisms with the pGDP2 plasmid, we would use a sniff test to ensure the production of our end-product, citronellol. The gene expression is constitutive, however the physical production of citronellol can be controlled through glucose presence, making the pathways induced by glucose. Levels of citronellol produced can be altered by controlling the yeast's exposure to glucose. As mass spectrometry technology is not available at the laboratory we plan on executing this experiment at, we hope to partner with other laboratories in the area for future testing to ensure the production of citronellol in our yeast. When working without mass spectrometry, rose petals and rose oil should be used as a comparative control to gage the degree of scent

produced by the transformed yeast when compared to usual baking supplements.

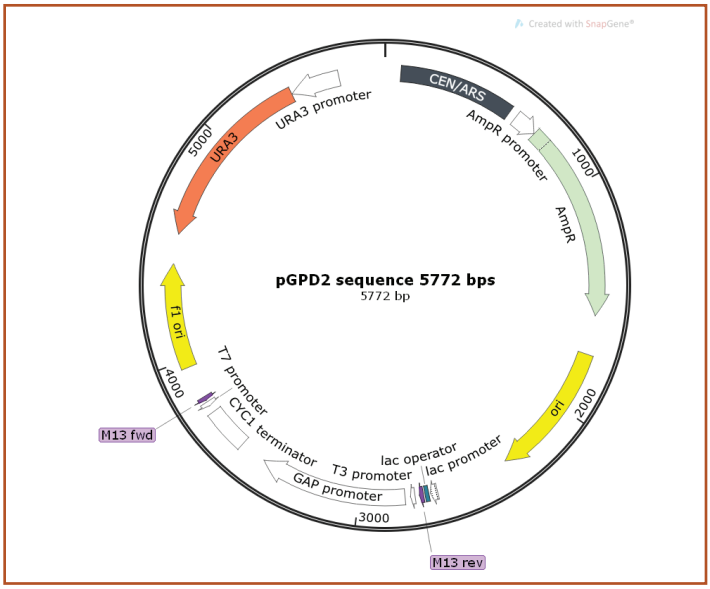

Figure 2. *This image shows the pGPD2 plasmid, the plasmid chosen to transform with our desired genes and insert into yeast. The plasmid is resistant to ampicillin for bacterial selection and URA3 for yeast selection. The plasmid features the GAP promoter and the CYC1 terminator (Crook, Freeman and Alper 2011). The desired genes will both be inserted between the GAP promoter and CYC1 terminator in one plasmid.*

Device level

The process of producing citronellol begins with glucose and undergoes three major steps. The Mevalonate (MVA) pathway and DXP reductoisomerase, two essential metabolic pathways, synthesize and transform dimethylallyl pyrophosphate and isopentenyl pyrophosphate (IPP and DMAPP, respectively) into Geranyl pyrophosphate, or GPP, as depicted in the first step in Figure 3. In the second step of the diagram, the enzyme geraniol synthase (GES) breaks down GPP and produces geraniol by removing two phosphate groups from GPP. Finally, The Old Yellow Enzyme (OYE) reduces an allylic alcohol double bond from geraniol, forming citronellol as depicted in the final step of the diagram (Yuan, Chen, Zhao, et al. 2011). The ScGES gene, which converts the GPP to geraniol, is derived from ObGES. The ScOYE gene, which consumes geraniol to produce citronellol, originates from HbOYE. Both new genes would be placed in the pGPD2 plasmid. This plasmid could either be transformed into a bacterium, then moved to yeast, or transformed into the yeast directly. We will codon optimize the genes for *S. cerevisiae* prior to assembly, as codon expression in plants differs from yeast expression.

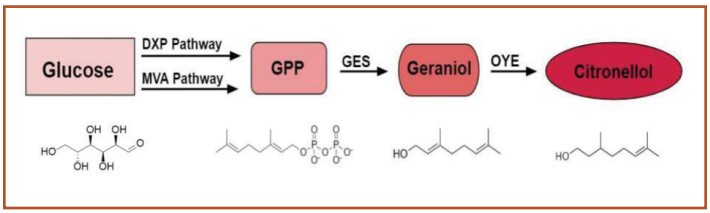

Figure 3. *The diagram depicts the pathway of glucose to citronellol, the product known for producing the classic rose scent. The process begins with glucose and undergoes three major steps to produce citronellol. Through the Mevalonate (MVA) pathway and DXP reductoisomerase (part of the MEP pathway), which are two essential metabolic pathways, DMAPP and IPP are synthesized and transformed into GPP. The enzyme geraniol synthase (GES) is used to produce geraniol from GPP by removing the two phosphate groups. Finally, with the Old Yellow Enzyme (OYE), an allylic alcohol double bond from Geraniol was reduced, and the final product, citronellol is produced (Yuan, Chen, Zhao, et al. 2011).*

Parts level

Our goal is to insert two genes, ScGES and ScOYE, into the plasmid pGPD2 (see Figure 2). This plasmid was chosen because the pGPD2 vector already possesses an appropriate promoter which functions in the presence of glucose, making it optimal for the pathways required in this work as glucose is a necessary part of the pathway. Most likely, for simplicity in the further steps of the experiment, we will transform the plasmid into *S. cerevisiae* and PCR the genes from the yeast. The plasmid contains the GAP promoter and CYC1 terminator with a multicloning site, so that the genes would be inserted in this location (Crook, Freeman and Alper 2011). The combination of ScGES and ScOYE within the pGPD2 vector will allow the organism transformed with the plasmid to produce geraniol and then citronellol, producing the rose scent. After sufficient safety testing, the transformed baker's yeast can directly be used for baking without any additional extraction methods, simplifying the flavored baking process.

We chose *S. cerevisiae* as the chassis for this synthetic biology project because our goal is to deliver an accessible yeast which may produce flavored or scented bread and baked goods. The *S. cerevisiae* should be purchased from Sigma Aldrich to ensure a pure yeast, as the eventual goal is to use the yeast for food production. *S. cerevisiae* is commonly referred to as baker's yeast because it is most often used for baking purposes, making it the most suitable organism for this paper's goals.

Safety

S. cerevisiae pose no safety issues as it is used commercially and is killed in the baking process.

Citronellol, the final product of our chemical process and the main component of the rose scent, poses minimal safety issues. The FDA considers it generally safe for ingestion (Environmental Protection Agency n.d.). A study done on rodents showed no adverse effects even at the highest dose of 10,000 ppm of geraniol tested within the experiment, therefore any remaining amount of geraniol not converted to citronellol should not pose a safety hazard (Baker and Grant 2018). Although rodent consumption does not equate to human consumption, this still indicates an initial safely. We do not currently have an approximation of how much citronellol the system would produce in parts per million, so this would need to be included as part of preliminary safety testing. In addition, geraniol and citronellol are commonly used in scented perfumes and other cosmetics, and has only been reported as hazardous to a small portion of the population with non-lethal allergies. As the compounds are used in perfumes, the allergies include allergies to the smell, however only a small portion of the population possesses these allergies (Baker and Grant 2018). Further safety evaluation would be necessary if this product were to go to the market for consumption purposes.

Discussions

Other considerations can be taken into account with this paper which could potentially improve efficiency and results. To maximize the efficiency of the citronellol production, several new genes could be introduced to the *S. cerevisiae*. One, ERG20, converts IPP and DMAPP from the MVA pathway into GPP. However, it also naturally converts GPP into FPP (which cannot be used in the citronellol production). A mutated version of ERG20 would hypothetically underproduce the FPP, directing the focus of the glucose conversion to GPP. Similarly, one can attempt to silence ERG20 using RNA interference to inhibit the expression of the gene (Tao, Li, Bolin, et al. 2018). This additional step bolsters production of GPP, which would allow for further production of geraniol and citronellol, increasing the capacity of the yeast to produce the rose scent.

If this work was successful and could be brought to the market, it would have the potential to revolutionize the flavored baking industry. Not only would it elevate the ease of producing intricate flavor and aroma infused baked goods, but it could ease the mass production of these goods by avoiding difficult processes and added costs of procuring supplemental extracts and flavors. In addition, procedures and genetic constructs developed in rose-infused yeast could be applied to a variety of other flavors, expanding the availability of the flavor industry in yeast. Please note that although no components of this synthetically engineered yeast pose a significant health hazard, significant further testing would be necessary before the yeast could be made available to the public. Although this product holds great potential, the public's perception of genetically modified organisms poses an issue when taking this product to market. In order to overcome this challenge, we would need to speak to proper advertisers and GMO health professionals with the capabilities of ensuring a genetically modified organism's safety for public consumption.

As this work moves forward, future researchers can proceed to study the efficiency of the protocol with other genes and compounds to advance this work and continually improve the system for optimal scent and flavor effect. This research is worth more than the traditional methods of extracting flavor or infusing flavor in baked goods, as it opens possibilities for eased production of baked goods in a variety of flavors beyond rose. In the long term, this work can improve cost efficiency on mass production of flavorful baked goods, as well as increase the availability of flavors.

Acknowledgements

The Western Reserve Academy BioBuilder Team would like to thank Dr. Pethel, our in-school mentor, for supporting us through the process of continuing our research from the previous year. In addition, we would like to thank Dr. Irene Reizman, our mentor given through BioBuilder, who greatly helped us locate all necessary parts for this project. Lastly, we would like to thank the MIT BioBuilder program for providing us with this opportunity, as well as for their help with locating online resources to advance our work.

This project was accomplished through participation in the BioBuilderClub, an after-school program organized by BioBuilder Educational Foundation. BioBuilderClub engages high school teams around the world to combine engineering approaches and scientific know-how to design/build/test their own project ideas using synthetic biology.

References

Baker BP and Grant JA. Geraniol profile [Internet]. Geneva (NY): New York State IPM Program; 2018 [cited 2020 Apr]. Available from: https://ecommons.cornell.edu/handle/1813/56127

Bender B. How long does it take to grow roses? [Internet]. Santa Monica (CA): Leaf Group Ltd., Hunker; 2020 [accessed 2020 May 28]. Available from: https://www.hunker.com/12194850/how-long-does-it-take-to-grow-roses

Crook NC, Freeman ES and Alper HS. Re-engineering multicloning sites for function and convenience. Nucleic Acids Res [Internet]. 2011 Aug [cited 2020 Apr 22];39(14):e92. Available from: https://www.ncbi.nlm.nih.gov/pubmed/21586584

Environmental Protection Agency. CITRONELLOL Biopesticides Registration Action Document: CITRONELLOL [Internet]. Washington (DC): US Environmental Protection Agency, Office of Pesticide Programs; [cited 2020 Apr]. Available from: https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/decision_PC-167004_11-May-04.pdf

Great British Chefs. How to cook with rose petals [Internet]. Great British Chefs; 2020 [cited 2020 Apr 20]. Available from: https://www.greatbritishchefs.com/how-to-cook/how-to-cook-with-rose-petals

Katsukawa M, Nakata R, Koeji S, Hori K, Takahashi S and Inoue H. Citronellol and geraniol, components of rose oil, activate peroxisome proliferator-activated receptor α and γ and suppress cyclooxygenase-2 expression. Biosci Biotechnol Biochem [Internet]. 2011 [cited 2020 Apr 21];75(5):1010-2. Available from: https://www.ncbi.nlm.nih.gov/pubmed/21597168

Rodriguez A, Strucko T, Stahlhut SG, Kristensen M, Svenssen DK, Forster J, Nielsen J and Borodina I. Metabolic engineering of yeast for fermentative production of flavonoids. Bioresource Technology [Internet]. 2017 December [cited 2020 May 2];245(B):1645-54. Available from: https://www.sciencedirect.com/science/article/pii/S0960852417309306

Tao W, Li S, Bolin Z, Bi C and Zhang X. Engineering *Saccharomyces cerevisiae* for the production of the valuable monoterpene ester geranyl acetate. Microbial Cell Factories. 2018 December;17(1):85.

Yuan T-T, Chen Q-Q, Zhao P-J, Zeng Y, Liu X-Z and Lu S. Identification of enzymes responsible for the reduction of geraniol to citronellol. Natural Products and Bioprospecting. 2011 Dec 13;1:108–11. doi: 10.1007/s13659-011-0032-6

Zymo Research. Frozen-EZ yeast transformation II kit [Internet]. (CA): Zymo Research; 2020 [retrieved 2020 Apr 26] Available from: https://www.zymoresearch.com/products/frozen-ez-yeast-transformation-ii-kit